The effect of particulate material and fatty acids on the anaerobic growth of Saccharomyces cerevisiae

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THE EFFECT OF PARTICULATE MATERIAL AND FATTY ACIDS ON THE ANAEROBIC GROWTH OF *SACCHAROMYCES CEREVISIAE*

Submitted by STEPHEN PATRICK HAGLEY

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SUMMARY

Trub, a fine particulate material that occurs in wort, was shown to stimulate fermentation of both brewer's wort and glucose-salts medium. This was manifested by more rapid attenuation, greater growth, greater suspended yeast counts and greater ethanol production than in controls. At high specific gravities the stimulative action was more pronounced in both types of media. Indeed, trub was a prerequisite for complete fermentation of glucose-salts medium, those fermentations from which it was absent being stuck. Addition of trub restarted stuck fermentations. The effect of trub has been attributed to either stimulation of yeast growth by nutrients, particularly sterols, unsaturated fatty acids and zinc ions, or alleviation of carbon dioxide-supersaturation and its attendant inhibition, through trub solids acting as nucleation sites for gas evolution.

Trub was shown to be rich in sterols, unsaturated fatty acids and zinc ions, all of which have potential to stimulate yeast fermentation. However, supplementation of glucose-salts medium with the ionic and lipid fractions of trub, or the trub solids resulting from such fractionation, did not stimulate fermentation to the same degree as trub. Supplementation of glucose-salts medium with linoleic acid and β-sitosterol, metal ions or activated charcoal did not prevent fermentations from sticking. Similar results were recorded in fermentations of brewer's wort. Conclusive evidence showed that yeast from fermentations of glucose-salts supplemented with trub took up lipids from the trub. It was also evident that a greater quantity of the $C_{18:2}$ fatty-acyl residue was present in yeast from fermentations of brewer's wort supplemented with trub than in yeast from control fermentations. The presence of a trub appeared to help keep yeast in suspension, indicating a physical role for trub's stimulative action. Evidence is presented to suggest that a deficiency in magnesium or zinc ions is not responsible for the poor performance of yeasts from control fermentations. It is also unlikely that trub's stimulative effect is due to the correction of a deficiency in any other ion. It is postulated that trub's stimulative action is brought about by a combination of effects,

one nutritive, through provision of unsaturated fatty acids and sterols, and the other physical, through provision of nucleation sites for carbon dioxide evolution which helps keep yeast in suspension.

The uptake of oleic acid under anaerobic conditions was studied in two strains of *Saccharomyces cerevisiae*, Y185 and X2180-1Aa. *Saccharomyces cerevisiae* Y185 incorporated over 60% of exogenously supplied oleic acid (30 mg l^{-1}), when harvested at mid-exponential phase, whereas *Saccharomyces cerevisiae* X2180-1Aa had incorporated over 70%. Varying the quantity of oleic acid supplied to cultures of *Saccharomyces cerevisiae* Y185 did not affect the growth yield. However, decreasing the amount of oleic acid supplemented to cultures of *Saccharomyces cerevisiae* X2180-1Aa decreased the growth yield, presumably by decreasing the time that cultures were in the exponential phase of growth.

Lipid extracts from cells of anaerobically-grown *Saccharomyces cerevisiae* Y185 contained a high proportion of free oleic acid. When cells were converted to sphaeroplasts over a period of 1 h there was transfer from free oleic acid to triacylglycerols and sterol esters. A similar conversion took place on converting cells of *Saccharomyces cerevisiae* X2180-1Aa to sphaeroplasts, however, the transfer was from phospholipids to triacylglyerols. Inhibitors of microtubule assembly and protein synthesis were unable to arrest the intracellular transfer of lipids in *Saccharomyces cerevisiae* Y185. The problems encountered with the occurrence of intracellular lipid metabolism during sphaeroplast formation need to be addressed if the processes by which fatty acids are taken up and incorporated into membranes by *Saccharomyces cerevisiae* are to be elucidated.

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GENERAL INTRODUCTION

Man has exploited yeasts for production of alcoholic beverages and foodstuffs over many centuries. Yeasts were used to produce a potable alcoholic beverage as long ago as 5000 B. C. Bread-making and brewing share common origins; indeed the earliest recorded brewing process is very similar to that used in the baking of bread. From these early times, brewing evolved as an art and a craft in the absence of any scientific knowledge. Improvements in beer quality and yeast purity were arrived at empirically. It was not until 1680 that Antonie van Leeuwenhoek observed yeast cells in drops of fermenting beer and wine using a primitive microscope. However, the nature of the yeast and its role in fermentation were not recognised until the Nineteenth Century. The concept of a causal relationship between alcoholic fermentation and yeast activity was first proposed in 1837. Cagniard-Latour, Kützing and Schwann, working independently, and mainly on the basis of microscopic observations of fermenting brews, advanced the theory. These reports, however, were ridiculed by senior members of the scientific fraternity of the time, who favoured a purely chemical explanation for fermentation, and it was not until the work of Pasteur, later in the century, that the role of yeast in fermentation was finally proven. Pasteur's two major works in the field, Études sur le Vin (1866) and Études sur la Bière (1876), solved the controversy. In the latter publication, Pasteur concluded that yeast cells cause fermentation when growing under anaerobic conditions, and that during the fermentation yeast converts sugar into ethanol and carbon dioxide. Further research at the beginning of the Twentieth Century by Embden, Meyerhof and Parnas elucidated the metabolic pathway by which yeasts catabolise glucose to ethanol and carbon dioxide. In the years since this discovery, research into the biochemistry, genetics and metabolism of yeast has allowed greater exploitation of these organisms for production of alcoholic beverages.

In more recent years, however, yeasts have begun to be seen in a different light, namely as tools for understanding the behaviour of cells, rather than solely as producers of alcoholic beverages. They are widely regarded as model eucaryotes in the study of cell biology, in particular *Saccharomyces cerevisiae* the most commonly

studied yeast species (Rose, 1980 a; Rose and Harrison, 1989). The eucaryotic nature of yeasts means that information concerning the molecular events that take place in them is likely to be relevant to cells of higher organisms. Many yeast genes have been mapped making it possible to relate genetics, cell physiology and metabolism. Their ease of cultivation and manipulation have ensured their widespread use in cell biology.

Saccharomyces cerevisiae is a facultative anaerobe and, as such, is able to grow in both aerobic and anaerobic conditions. The yeast has a requirement for molecular oxygen for the synthesis of sterols (Klein, 1955) and unsaturated fatty-acyl residues (Bloomfield and Bloch, 1958). Andreasen and Stier (1953, 1954) first demonstrated the auxotrophic requirement of *Saccharomyces cerevisiae* for exogenous sources of these two lipids when yeast was grown under anaerobic or oxygen-limited conditions.

Biosynthesis of sterols can be divided into three parts. These are mevalonate synthesis; conversion of mevalonate to the open-chain isoprenoid hydrocarbon squalene; and cyclisation of squalene to give lanosterol and other sterols (Hunter and Rose, 1971; Ratledge and Evans, 1987). The first two steps can be performed by *Saccharomyces cerevisiae* under anaerobic conditions (Klein *et al.*, 1954). However, molecular oxygen is required by squalene mono-oxygenase to catalyse formation of squalene epoxide, an intermediate in the conversion of squalene to lanosterol (Fryberg *et al.*, 1973; Jahnke and Klein, 1983). The intermediate undergoes cyclisation to lanosterol. Lanosterol is converted to other sterols by dehydration and desaturation reactions some of which also require molecular oxygen (Popjak and Cornforth, 1960).

Unsaturated fatty acid synthesis occurs in *Saccharomyces cerevisiae* from preformed saturated fatty-acyl CoA esters. The substrates for desaturation reactions are long-chain (principally $C_{16:0}$ and $C_{18:0}$) fatty-acyl CoA esters. A desaturase introduces a double bond at the Δ^9 position producing palmitoleyl-CoA from pamitoyl-CoA and oleyl-CoA from steroyl-CoA. Desaturase activity is dependent upon molecular oxygen (Bloomfield and Bloch, 1958, 1960). Anaerobically-grown

Saccharomyces cerevisiae has been shown to have low desaturase activity and evidence suggests that lack of oxygen completely inactivates the enzyme (Klein and Volkman, 1975).

Therefore, under strict anaerobic conditions, *Saccharomyces cerevisiae* has a requirement for a sterol (Bloch 1983) and an unsaturated fatty acid (Jollow *et al.*, 1968; Bulder and Reinink, 1974) supplements. Both requirements are fairly broad (Light *et al.*, 1962; Proudlock *et al.*, 1968; Hossack and Rose, 1976). Large quantities of free sterol are produced from hydrolysis of sterol esters (Aries and Kirsop, 1978). Also, during anaerobic growth, cells accumulate large amounts of squalene and, on exposure to oxygen, rapidly convert this precursor into sterols (Jollow *et al.*, 1968; Klein, 1955). The chain length and degree of unsaturation of fatty-acyl residues in phospholipids are affected if yeasts are grown anaerobically (Jollow *et al.*, 1968). However, David (1974) has shown that the requirement for molecular oxygen in yeast is greater for biosynthesis of sterols than for unsaturated fatty-acyl residues.

Researchers have exploited the anaerobically-induced requirement of *Saccharomyces cerevisiae* for lipid supplements to conduct composition-function studies on the yeast plasma membrane. The effects of fatty-acyl unsaturation on wall and membrane biogenesis (Alterthum and Rose, 1973), solute transport (Keenan and Rose 1979; Keenan *et al.*, 1982; Prasad and Rose, 1986), the ability of membranes to resist stretching (Hossack and Rose, 1976) and the effects of membrane composition on ethanol tolerance in yeasts (Thomas *et al.*, 1978; Thomas and Rose, 1979) are all examples of such composition-function studies.

However, some strains of *Saccharomyces cerevisiae* have been shown to grow under anaerobic conditions in the absence of a sterol and an unsaturated fatty acid (Macy and Miller, 1983). However these yeasts had a prolonged lag phase of growth, and provision of Tween 80®(a source of oleic acid) and ergosterol significantly stimulated anaerobic growth. Bulder and Reinink (1974) demonstrated that *Schizosaccharomyces japonicus* has an anaerobic pathway for synthesis of unsaturated fatty acids. However the growth system may not have been fully

anaerobic and the enzymes involved may have an abnormally high affinity for minor traces of oxygen.

Although traditionally regarded as anaerobic, alcoholic fermentation has been shown to be stimulated by trace amounts of oxygen for the reasons described earlier, namely that molecular oxygen is required as a yeast growth factor (Akbar*et al.*, 1974) for synthesis of sterols and unsaturated fatty acids. The effect of oxygen on yeast fermentations has been demonstrated. Addition of oxygen stimulated fermentation rate, whereas removal of oxygen inhibited fermentation. Oxygen deficiency retards yeast growth (David and Kirsop, 1973) and, as such, although yeast cells may survive anaerobically during batch fermentations of beer, molecular oxygen must at some point be provided if fermentation is to proceed satisfactorily (Kirsop, 1978). Fermentative activity of yeast cells does not appear to be affected by oxygen deficiency. The decrease in fermentative activity of the final cell population in a fermentation is simply due to less viable yeast being present.

The oxygen requirement for growth of yeast cells, particularly in fermentations, has been described in terms of an oxygen credit or charge (Kirsop, 1974; Rose, 1978). Three components make up the oxygen credit: (1) molecular oxygen dissolved in the wort (oxygen is sometimes sparged into the wort prior to fermentation); (2) the content of cellular components in the pitching yeast that require molecular oxygen for synthesis; (3) the content in the fermentation medium of preformed components that require molecular oxygen for their synthesis (nicotinic acid, sterols and unsaturated fatty acids). The dissolved oxygen component of the oxygen credit is rapidly utilised. To synthesize further sterols and unsaturated fatty acids for incorporation into phospholipids, the intracellular reserves of sterol esters and triacylglycerols must be tapped. The utilisation of reserves in this way enables yeast to reproduce (Kirsop, 1974). However, the oxygen credit can be consumed to such an extent that yeast growth can be limited, with subsequent alterations in metabolism affecting the flavour of the final beverage (David, 1974; David and Kirsop, 1973; Haukeli and Lie, 1976). Evidence that components of brewer's wort add

to the oxygen credit has been reported (David and Kirsop, 1972, 1973) in experiments where wort sterols and unsaturated fatty acids were incorporated into yeast cells. These reports also explained the findings of other workers in which yeast, harvested from malt-wort fermentations, contained linoleyl residues, whereas yeast from defined medium fermentations did not (Suomolainen and Keranen, 1968). The uptake of polyunsaturated fatty acids, principally linoleic acid, from wort not only increases the oxygen credit in the yeast, but may also increase its ethanol tolerance. This possibility has led to interest in the lipid composition of brewery worts. In addition to wort lipids other components of brewer's wort which may affect yeast fermentation performance, such as amino acids, trace metals and fine particulate material, have become topics for research. Investigations into the affect of particulate material on fermentations and the uptake of fatty acids by *Saccharomyces cerevisiae* are dealt with in this Thesis, which is divided into two parts.

PART ONE: ROLE OF PARTICULATE MATERIAL IN BREWERY FERMENTATIONS

INTRODUCTION

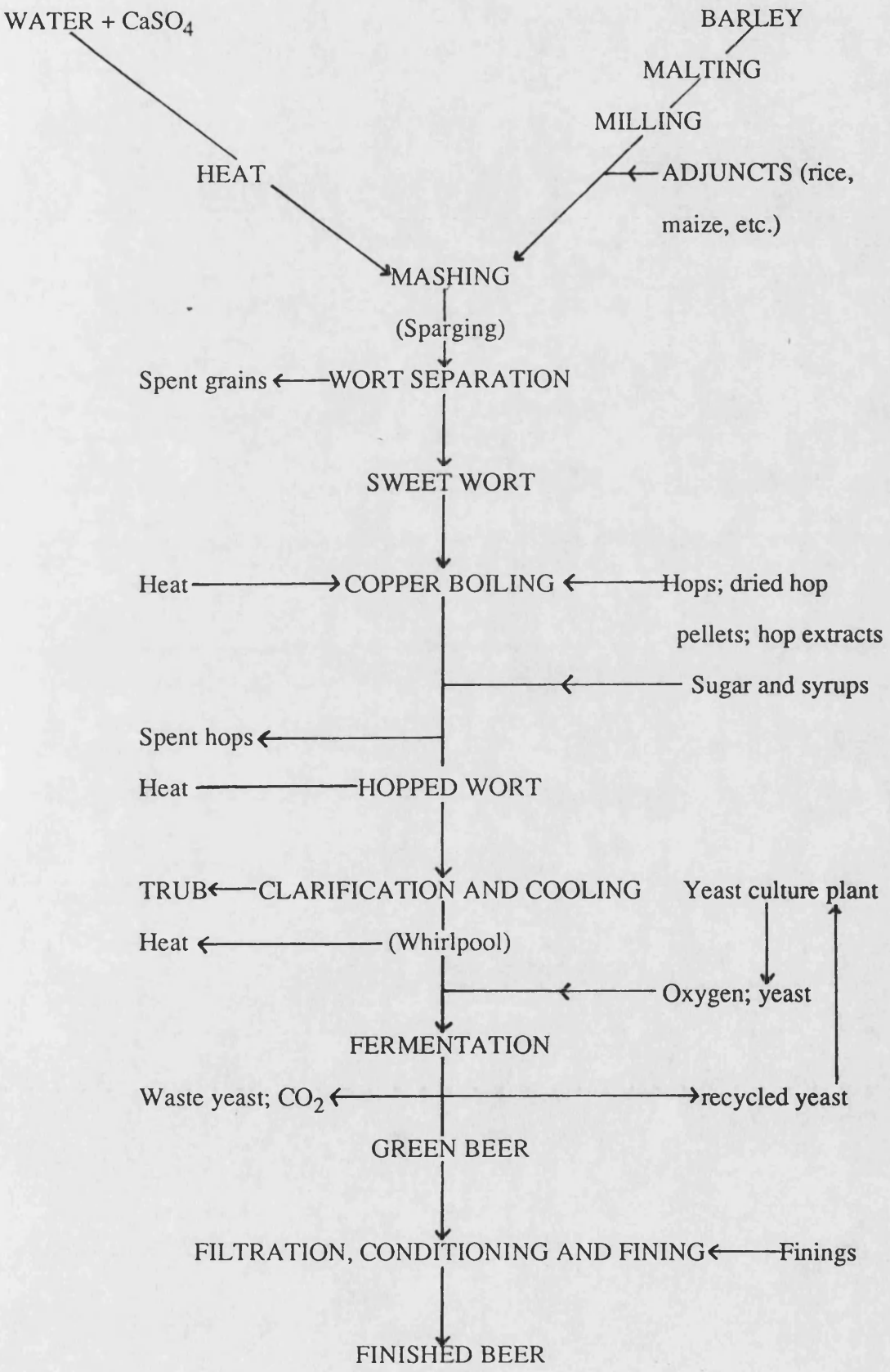
The Pocket Oxford Dictionary definition of beer as "an alcoholic beverage made from fermented malt, etc., flavoured with hops, etc.," succinctly names, either directly, or by implication, the four major ingredients of beer, namely water, malt, hops and yeast while leaving scope for the inclusion of other components. The stiochiometry of the conversion, by yeast, of simple sugars to yield ethanol and carbon dioxide is described in the Gay-Lussac equation:

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$$

and the sequence of biochemical processes which prosecute this conversion are known as the glycolytic pathway. The production of beer involves a number of fundamental operations which are illustrated in Figure 1.

Barley is the basic raw material for brewing. Barley acts as a source of amino acids and fermentable sugars upon which yeast grows to produce ethanol. However,these components are in polymeric storage forms in the barley seed and must be rendered soluble, by a process known as malting, for fermentation to occur. During malting, barley seed is steeped in aerated water at a temperature of approximately 16°C to induce germination in the grains. The grains are normally steeped for about two days, during which their moisture content increases to approximately 45%. Grains are then drained and "cast" into open-topped compartments where humidified and attempered air is passed through slots at the base. The use of helical turning devices ensures even distribution of the humidified air. Germination proceeds for up to five days. In response to water and oxygen, the embryo secretes the hormone gibberellic acid to the aleurone cells (MacLeod and Miller, 1962; MacLeod and Palmer, 1966; Palmer, 1974). Gibberellic acid induces the synthesis of a number of hydrolytic enzymes; endo-β-glucanase, α-amylase (β-amylase being present in the seed) and peptidases. The endosperm cell walls which contain insoluble and soluble β-linked glucan, amongst other components, are

FIGURE 1. The Brewing Process



degraded by the action of endo-β-glucanase, possibly in conjunction with other enzymes (Bamforth, 1981). These cells contain starch granules embedded in a protein matrix of hordein. The starch and hordein are subsequently degraded by amylases and peptidases, respectively. However, the major action of the amylases takes place during mashing and therefore measures are taken to prevent complete solubilization of storage compounds. As a consequence malting allows the grain to germinate and limits the amount of seedling growth. Further growth is prevented by drying the grains in a kiln. Grains are dried with hot air at 65-80°C, which reduces the moisture content to less than 3%. The seedling is killed, as are any micro-organisms on the surface of the grain, by the heat, which also promotes the formation of aromatic colour and flavour compounds, such as the melanoidins. Kiln-drying is essential if grains are to be stored, but, as a consequence of drying, a certain proportion of the hydrolytic enzymes are inactivated, between 4 and 30% depending on the enzymes and the kilning schedule.

Following kilning the malt is transferred to the brewery, milled and mixed with hot water in the mash tun. The ionic components of the water, particularly the bicarbonate ion and calcium ion content, are important in determining the pH, which is vital to a successful mash, in the mash tun. Generally bicarbonate ions are removed by lime-treatment or ion-exchange resins and calcium ions, through a series of ionic interactions, give rise to a pH of about 5.4 in the mash tun. If the calcium ion content of the water is insufficient, $CaSO_4$ is added to attain the required pH value. A pH value of about 5.4 represents a compromise between the pH optima of the various hydrolytic enzymes, and thus yields the maximal fermentable extract from the malt. The object of mashing is to allow malt enzymes to convert starch into dextrins and fermentable sugars, and proteins into amino acids and peptides. Mashing also renders soluble a variety of other components including tannins, inorganic salts and vitamins. The activity of α-amylase in the malt is more than sufficient to degrade its the starch reserves. The importance of this is realised when one considers the use of cheap unmalted cereal adjuncts (wheat, maize and rice) which do not contain the enzyme.

There are two basic systems for mashing: (1) infusion mashing; (2) decoction mashing. Infusion mashing, which evolved in Britain over many centuries, is characterised by a low ratio of water : grist, a uniform mashing temperature (about 65°C) and the use of a single vessel for extraction and filtration. This gives rise to a thick mash in which the grist floats to the surface. Soluble sugars, amino acids and vitamins diffuse out of the grist causing the specific gravity of the liquid to rise. After a stand of one to two hours, the sweet wort is drawn off through slots in the bottom of the mash tun and the grains are washed, or sparged, with treated water at about 70°C to remove residual soluble materials. In decoction mashing, the ratio of water : grist is higher, the initial mashing temperature lower (about 35°C), the overall mash time longer than for infusion mashing, and separate vessels are used for extraction and filtration. Portions of the decoction mash are removed, heated to a higher temperature, then replaced, gradually raising the temperature of the whole mash until it reaches as high as 75°C. When mashing is complete the mash is pumped to a separate vessel, the lauter tun, where sweet wort is filtered off and the grains sparged.

Infusion mashing takes place at a temperature which, although α-amylase is active, inactivates proteinases, β-glucanases and β-amylase. However the coarse nature of the malt and the thick mash is thought to confer some amount of protection to these enzymes. With decoction mashing, the low initial temperature stand favours activity of heat-labile enzymes such as proteinases (Pollock, 1988), β-glucanases and β-amylase. The removal, heating and replacement of portions of the mash acts to stir the thin mash of the decoction system, thereby favouring separation of differently sized particles. The infusion mash is generally associated with English ales, whereas the decoction mash is generally used for lager production. It has been reported that changes in the mashing process can have marked effects on beer properties (Hudson, 1973).

The sweet wort obtained from the mash is passed into a vessel called the copper and is boiled, together with dry hops or hop pellets, plus any additional sugars or syrups. Wort-boiling lasts for one to two hours and serves to sterilize the wort,

inactivate enzymes that have survived mashing, denature and precipitate proteins, dissolve additional sugars, isomerise hop α-acids and, volatilise and remove unwanted flavour compounds. The most important reaction is the conversion of α-acids to iso-α-acids which are responsible for the bitter flavour of beer. Amongst the flavour components from hops are humulene and myrcene. Following wort-boiling, hops are separated from the wort in a hop-strainer and the remaining precipitate or trub is removed by a whirlpool tank or by filtration (MacLeod, 1977). The removal of trub from wort is discused in greater detail later in this review. The wort is then cooled by a heat exchanger, diluted to the required specific gravity, aerated and transferred to the fermentation vessel. Wort is a complex growth medium for yeast. A typical profile of the carbohydrates, weight by volume, would be: 1.0% glucose; 3.9% maltose; 1.1% maltotriose; 0.2% maltotetraose; 2.3% higher dextrins plus traces of sucrose and fructose (Harris *et al.*, 1951). The use of sugar syrups can alter this profile (MacLeod, 1977).

FERMENTATION OF WORT

The yeast *Saccharomyces cerevisiae* is used in brewery fermentations and yeast strains may be divided into two groups depending on slightly different characteristics: top-fermenting yeast and bottom-fermenting yeast. Top-fermenting yeasts are generally used in ale fermentations, whereas bottom-fermenting yeasts are used in lager fermentations and tend to settle in the bottom of the fermenting vessel. The two types of yeast were once regarded as being taxonomically distinct on the grounds that the lager yeasts (formerly *Saccharomyces uvarum*) are able to ferment the trisaccharide raffinose, due to the production of melibiase, whereas ale yeasts can only ferment one-third of this molecule. Nowadays, however, both ale and lager yeasts are regarded as *Saccharomyces cerevisiae*.

In the traditional batch brewery system, yeast is collected from a previous fermentation and is added to wort. The pitching rate, or inoculum, is dependent upon

the type of beer being brewed and the yeast strain used, and is generally greater for ale fermentations than lager fermentations. Ale fermentations are traditionally performed at 15°C, whereas lager production occurs at a lower temperature (8°C). Biochemical events in the ale fermentation are virtually complete after two to three days, whereas lager fermentations, due to the low temperature, last for up to 14 days, although the general changes occurring in the wort are common to both. Uptake of amino acids (Jones and Pierce, 1964) and carbohydrates follows a sequential pattern. As the yeast converts the carbohydrates into ethanol and carbon dioxide, via the glycolytic pathway, a decrease in specific gravity occurs, a process known as attenuation. In addition to the wort nutrients, oxygen is required for the synthesis of essential lipids (see General Introduction).

During fermentation, yeast synthesizes many metabolic by-products some of which have a disproportionate effect on the flavour of the finished beer. These by-products include higher alcohols, fatty acids and esters. The higher alcohols (2- and 3-methylbutanol, iso-butanol, *n*-propanol and 2-phenylethanol) impart a fragrant aroma and are formed from amino acids (Äyräpää, 1971; Inoue, 1975). Fatty acids, particularly hexanoic (C_6), octanoic (C_8), decanoic (C_{10}) and dodecanoic (C_{12}), are synthesized by the yeast during fermentation (Taylor and Kirsop, 1977) and impart a "soapy-fatty" flavour (Clapperton and Brown, 1978). Esters formed during fermentation are associated with "aromatic", "fragrant", "fruity" and "floral" flavours and are sub-grouped as "estery" (Clapperton *et al.*, 1976; Meilgaard *et al.*, 1979).

At the end of primary fermentation, the majority of the yeast is removed by a natural phenomenon known as flocculation, where cells clump together. Conditioning of the beer then takes place. During conditioning, certain volatiles, such as acetaldehyde, diacetyl and hydrogen sulphide are removed. The beer is carbonated and the yeast cells separate to yield a bright beer. For traditional cask ales this process is allowed to occur naturally at low temperature. The removal of diacetyl form beer by enzymes within the yeast is regarded as the rate-limiting step in conditioning. Residual yeast and particular material are removed from conditioned beer by

centrifugation or filtration, usually through kieselguhr deposited on a support. Precipitation of chill-haze material occurs on cooling and the beer is then artificially carbonated. Storage of beers, for long periods, necessitates pasteurisation of the beverage. Many draught beers are flash-pasteurised (20 sec; 70°C) while others are tunnel-pasteurised, where the containers are sprayed with hot water for up to one hour. The advantage of beer stability gained through pasteurisation can be compromised by damage to flavour components.

HIGH-GRAVITY BREWING

It has been the an accepted practice in North America to brew and ferment worts of a relatively high specific gravity and then dilute the beer to the required alcoholic content for sale (Schaus, 1971). There are a number of advantages with high-gravity brewing which include: increased plant efficiency (Whitear and Crabb, 1977; Hackstaff, 1978); reduced energy and labour costs (Hackstaff, 1978); use of higher adjunct ratios, reducing raw material costs (Pfisterer and Stewart, 1975); improved smoothness of taste and general flavour stability (Pfisterer and Stewart, 1975); and improved colloidial stability leading to longer shelf life (Whitear and Crabb, 1977). The major advantage is the first one mentioned above. It means that increased demand for beer can be met by existing plant brewing the same volume of beer at a higher specific gravity and diluting the product. This requires minimal capital costs because new plant is not required. However, the disadvantages of high-gravity brewing, such as poorer extract yield, poorer hop-utilisation and poorer nutrient quality through the use of corn-syrup adjuncts (Casey *et al.*, 1984), can combine to negate the positive effects. In addition, a major capital cost is incurred in the preparation of the dilution water. Amongst the requirements for the dilution water are: sterility; that it be free from particular material (i. e. bright); pH value at the same level as that of the beer; carbonation to the level of the recipient beer; free from oxygen (< 0.1 p.p.m.) and chlorine (< 0.02 p.p.m.); and free from heavy metal ions

such as copper and iron (Thurston *et al.*, 1982).

The flavour of the product can be affected when yeast ferments a high-gravity wort. Problems have been reported with an unacceptably high "fruity" aroma, associated with ethyl acetate and iso-amyl acetate, which can be overcome, to some extent if the wort specific gravity is kept below 1.060 (Whitworth, 1978).

WORT CONSTITUENTS AND THEIR EFFECTS ON FERMENTATIONS

In addition to the amino-acid and carbohydrate constituents of wort, referred to earlier in this section, there are other minor components of the wort whose presence is vital to yeast fermentation performance. These components include: inorganic metal ions; lipids and oxygen; purines; and vitamins. Brown and Kirsop (1972) reported that all minor essential wort constituents, other than amino acids, are normally present in an all-malt wort in a twofold excess.

Lipids and oxygen

Oxygen is a prerequisite for synthesis of sterols and unsaturated fatty acids in yeast. Normally wort is aerated prior to pitching of the yeast. The dissolved oxygen is rapidly consumed by the pitching yeast (Kirsop, 1974). The lipid composition of the pitching yeast is important because, if it is deficient in sterol or unsaturated fatty acid, yeast growth is prevented and fermentation slows or may be completely arrested (Ahvenianen, 1982).

Fatty acids

In recent years a number of studies have investigated the occurrence of lipids through the stages of the brewing process (Anness, 1984; Anness and Read, 1985 a, b; Cantrell and Anderson, 1983; Jones *et al.*, 1975; O'Palka *et al.*, 1987). In quantitative terms, lipids are relatively minor components of the raw materials used in brewing (Anness, 1984). Malts contain an estimated 3% lipid on a dry weight basis and cereal

adjuncts rarely contain more than 4%. The amounts in hops and hop pellets, though largely dependent upon seed content, are usually less than 4-5% of dry weight (Anness and Read, 1985 a). While lipids are not readily extracted from these raw materials during wort production (Anness and Read, 1985 a), the quantities that are present in wort are sufficient to affect yeast performance (Ahvenianen, 1983) and provide a considerable reserve of material that is potentially deleterious to beer flavour (Drost *et al.*, 1971). The majority of malt and hop lipids are retained in the spent grains and spent hops, respectively (Anness and Read, 1985 a).

Determination of wort lipids is best achieved by mild acid hydrolysis of freeze dried wort prior to solvent extraction (Anness and Read, 1985 a). Lipids can then be determined as total long-chain fatty acids by gas chromatography analysis of fatty-acyl methyl-esters (Anness, 1984; Morrison *et al.*, 1980). Such techniques are necessary since small quantities of lipids can affect yeast metabolism, (Ahvenainen, 1983) and the foaming qualities of wort and beer (Bamforth and Jackson, 1983; Carrington *et al.*, 1972). Jones *et al.* (1975) showed that the predominant free fatty acids found in a wort of specific gravity 1.035 were $C_{16:0}$ and $C_{18:2}$. Over 96% of the free fatty acids, both saturated and unsaturated, found in wort were C_{14} to C_{18}. MacPherson and Buckee (1974) showed, for ale worts of the same specific gravity and composition, that the method of wort separation affected the results. Higher levels of $C_{16:0}$ and $C_{18:2}$ were found in worts obtained by centrifugation as opposed to mash-tun separation.

Chen (1980) demonstrated that the fatty-acid composition of the growth medium changed markedly during the course of beer fermentation. Palmitic ($C_{16:0}$), linoleic ($C_{18:2}$), stearic ($C_{18:0}$) and oleic ($C_{18:1}$) acids comprised 85-90% of wort fatty acids, whereas the medium-chain fatty acids, hexanoic ($C_{6:0}$), octanoic ($C_{8:0}$) and decanoic ($C_{10:0}$), accounted for 75-80% of beer fatty acids. These changes supported the findings of other workers (Klopper *et al.*, 1975). Chen used radiolabelled palmitic, oleic and linoleic acids to trace wort fatty acids through the fermentation. Up to 75% of the radiolabel was incorporated into the free fatty acids

and phospholipids of the yeast. Most of the palmitic was desaturated to $C_{16:1}$, whereas $C_{18:1}$ and $C_{18:2}$ fatty acids were further desaturated. None of the radioactive fatty acid was detected in fatty acids of the final beer, and a route for degradation of long-chain wort fatty acids by yeast to yield medium-chain length fatty acids was regarded as unlikely.

Fatty acids can have both advantageous and detrimental effects on yeast fermentation performance and beer quality. In general, long-chain polyunsaturated fatty acids are beneficial whereas short- and medium-chain length fatty acids are detrimental. The advantageous effects of long-chain unsaturated fatty acids, either already present in the wort or supplemented during fermentation, were first demonstrated in saké fermentations (Hayashida *et al.*, 1974, 1975; Hayashida and Ohta, 1980). Hayashida and his co-workers identified a proteolipid supplement which helped saké yeast attain high alcohol concentrations, up to 20% (w/v). The existence of similar "survival factors" was reported in high-sugar grape musts (Lafon-Lafourcade *et al.*, 1979). Ohta and Hayashida (1983) went on to demonstrate that Tween 80®(a source of oleic acid) had a role in the enhancement of ethanol tolerance in saké yeast. Similar success with the use of lipid supplements to increase ethanol tolerance has been reported in beer (Casey *et al.*, 1983, 1984) and wine fermentations (Ingledew and Kunkee, 1985). These findings are discussed in greater detail later in this review.

In addition to increasing the ethanol tolerance of yeast and the ethanol yield of a fermentation, unsaturated fatty acids, in worts and in enriched yeast cells, are known to decrease synthesis of volatile esters (Thurston *et al.*, 1981; Anderson and Kirsop, 1974, 1975). Esters impart a fruity or solvent-like aroma which can be undesirable, especially in high-gravity brewing (Ahvenainen, 1982). Linoleic acid is the most efficient inhibitor of ester synthesis and oleic acid is also effective (Äyräppä and Lindstrom, 1977; Thurston *et al.*, 1981). Therefore, some fatty acids have a beneficial effect on yeast metabolism, permitting production of high levels of ethanol under anaerobic conditions, increased tolerance to the toxic effects of ethanol and decreased

production of some flavour-active compounds. The final concentrations of fatty acids and their esters in beers is determined by a number of factors such as, oxygen tension, pitching rate, temperature and yeast strain (Wackerbauer and Bender, 1983).

The detrimental effects of fatty acids are demonstrated towards the end of fermentation and in the final beer. Generally these effects are attributed to short- and medium-chain fatty acids. Short- and medium-chain fatty acids may account for 85-90% of total beer fatty acids. They impart a caprylic or soapy/fatty flavour to beers (Maschelein, 1981). Fatty acids produced by yeast during fermentation of a grape must were found to have an inhibitory effect on fermentation (Lafon-Lafourcade *et al.*, 1984). These workers identified decanoic and octanoic acids and their corresponding ethyl esters as the factors which, acting synergistically with ethanol, inhibited yeast growth. They also found that the addition of yeast ghosts, i. e. that part of the cell remaining after yeast extract has been removed, eliminated the inhibitory effect. It was postulated that yeast cell hulls absorbed the fatty acids and esters responsible for inhibition of fermentation allowing it to proceed normally. Other workers have substantiated these findings (Ribéreau-Gayon, 1985; Munoz and Ingledew, 1989 a, b). It would appear from these reports that yeast ghosts work in two ways. Firstly they absorb potentially toxic medium-chain fatty acids produced by the yeast and, secondly, they act as a source of lipids which may be used by the yeast to prolong cell growth and viability. Wort fatty acids can have an adverse effect on the foaming qualities of wort and beer (Bamforth and Jackson, 1983).

Sterols

The importance of yeast sterol metabolism during brewery fermentations has been the subject of a number reports over years. David (1974) stated that the prime requirement of yeast for oxygen was for synthesis of sterols. Indeed the appearance and composition of sterols in yeasts during fermentation seems to be directly linked to the availability of oxygen (Aries and Kirsop, 1977). When cells depleted of sterol are added to an aerated wort sterols are rapidly synthesized, increasing the sterol content

of the yeast, until all of the oxygen has been utilised. After this point has been reached, sterol levels decline, in response to the cessation of sterol synthesis, until a limiting concentration is arrived at when reproductive growth is inhibited. Sterols are stored as sterol esters outside membranes in yeast grown in the presence of oxygen. Free sterols accumulate in membranes in the absence of oxygen. The amount of sterol synthesized in the presence of oxygen determines the extent of growth in yeast. It has been reported that pitching rate can influence the extent of sterol synthesis (Aries and Kirsop, 1977; Kirsop, 1977, 1978). Wort-derived sterol does not appear to be as significant a nutritive factor as wort-derived fatty acids in growth and fermentation by yeast.

The analytical problems associated with extracting and isolating lipids from wort have exercised many workers. However, suitable methods now exist for analysis of medium- and long-chain fatty acids, and sterols in wort, beer and other beverages (Arkina, 1968; Zurcher, 1971; MacPherson and Buckee, 1974; Taylor and Kirsop, 1977; Loughrey and Letters, 1983; Hawthorne *et al.*, 1986; Stack *et al.*, 1986).

Oxygenation

The requirement for preformed sterols and unsaturated fatty acids by yeast during anareobic fermentation is a result of the lack of molecular oxygen. Although lipids may be supplemented to lipid-deficient worts on a laboratory scale, such a course of action is not open to the brewer because of the cost involved. However, brewer's can aerate their worts to overcome this problem. Two factors affect the solubility of oxygen in wort, namely, temperature and the specific gravity (Kruger, 1971). The concentration of dissolved oxygen is inversely proportional to the temperature and specific gravity of the wort. Therefore, a requirement exists for suitable aeration regimes to promote lipid biosynthesis for cell growth. These, in turn, help maintain cell viability and improve tolerance to ethanol, but do not in any way impair the quality of the product or its fermentation, which is essentially an anareobic process. Some workers have endeavoured to determine optimum aeration regimes for

alcoholic fermentation with regard to the lipid requirements of the yeast (Ohno and Takahashi, 1986 a, b). Most recently, O'Connor-Cox and Ingledew (1990) have demonstrated aeration of wort to be most stimulative 10-14 hours after pitching in fermentations of very high-gravity worts by a commercial lager yeast, *Saccharomyces uvarum* (*cerevisiae*). However, the oxygen requirements of *Saccharomyces cerevisiae* differ from strain to strain and at least four different groups can be identified, depending upon their oxygen requirement (Kirsop, 1974). Yeast strains with different oxygen requirements can ferment wort equally well if the aeration regimes are tailored to their individual needs. Therefore, the variations in oxygen requirement amongst yeast strains may be due to altered oxygen demand at different stages of growth. When yeast cells are propagated in the presence of oxygen, they will generally not require additional oxygen during fermentation (David and Kirsop, 1972, 1973). Furthermore when aerobically propagated yeast is pitched into non-aerated wort no more unsaturated fatty acids are synthesized and the lipid stores of the pitching yeast are distributed among the daughter cells (Ahvenianen, 1982).

Brewer's yeast can therefore be produced in forms which do and do not have a requirement for additional oxygen during fermentation. Whether cells have a requirement for oxygen depends on their oxygen credit derived during propagation. The method of yeast propagation can have a pronounced effect on beer flavour. The metabolic processes which produce certain flavours are dependent upon the reproductive activity of the yeast which, in turn, is ultimately linked to the oxygen credit carried by the yeast.

Yeast viability is often linked to oxygen availability; oxygen deficiency is often the cause for a decline in viability. In continuous fermentations, cell elongation may occur under certain conditions of aeration in some yeast strains (Brown and Hough, 1965). Alterations in the ratio of assimilable carbon to assimilable nitrogen can cause similar morphological changes (Brown and Hough, 1965); the ability of yeast to assimilate nitrogen is affected by the degree of oxygenation (Jones and Pierce, 1964).

Yeast pitching rate has been shown to have a profound effect on the progress of a fermentation. When pitching is at half the usual rate, problems arise with fermentation due to a decrease in the amount of new yeast and, as a consequence, a lesser degree of attenuation (Kirsop, 1978). Under conditions of high-gravity brewing, the problems outlined above are exacerbated (Casey and Ingledew, 1983). Early losses in yeast viability recorded in high specific-gravity worts were significantly decreased by pitching at higher than usual pitching rates. Kirsop (1978) reported that, at lower pitching rates, utilisation of wort-dissolved oxygen by yeast is inefficient and, as a result, cell-mass synthesis decreases, and the rate of fermentation declines. The inhibitory effects of excessive oxygen on yeast have been demonstrated (Gottlieb, 1971). The occurrence of such effects in brewery fermentations is unlikely, although David and Kirsop (1973) reported a yeast strain with a very low oxygen requirement which grew less rapidly in oxygen-saturated wort.

Oxygen is an important nutrient in alcoholic fermentations performed under brewery conditions. As such, it is essential for the brewer to establish an optimum oxygenation regime for his worts for each of the yeast strains he uses.

Metal ions with particular reference to magnesium and zinc

The importance of trace metal ions in the production of beer has long been recognised (Hudson, 1959). Small quantities of metal ions can have a profound effect on the metabolism of the yeast and the physical qualities of beer. Olsen and Johnson (1949) stated that, for *Saccharomyces cerevisiae* to grow in synthetic medium, there is a minimum requirement for 0.075 p.p.m. iron, 0.012 p.p.m. copper and 0.2 p.p.m. zinc. Generally much greater amounts are present in brewer's wort (Hudson, 1959). This study concentrated on two metal ions known to be essential for fermentative metabolism in yeast, namely, magnesium and zinc. The roles played by magnesium and zinc ions appear to be two-fold, namely, enzymatic or structural (Jones and Greenfield, 1984a).In enzymes, the ions serve as catalytic centres, activators or stabilisers of enzyme function, or maintain physiological control by antagonism of

activators and de-activators.

The precise role of zinc is not clear although it is known to be essential for the function of many enzymes including alcohol dehydrogenase, aldolase and glyceraldehyde-3-phosphate dehydrogenase (Vallee and Hoch, 1955; Coleman and Wiener, 1973; Van Engel, 1969), stimulates protein synthesis (Maddox and Hough, 1970), and carbohydrate metabolism (Visuri and Kirsop, 1970). Zinc ions can bind reversibly to anionic sites on the yeast cell membrane and be transported into the yeast cell (Fuhrmann and Rothstein, 1968). The rate of uptake is decreased at low pH values.

Brewer's wort, as stated earlier, generally meets the ionic requirements for yeast growth. However, some zinc deficient worts have been reported (Stone, 1962). The deficiency in zinc ions was due to their being retained by the insoluble matter in the mash. Many malt and wort components exhibit an ability to bind metal ions. Examples include amino acids, particularly cysteine and histidine, polypeptides, polyphenols, and phytic acid. These components must therefore be regarded as playing an important role in metal partition in brewer's wort (Lie *et al.*, 1975). The level of zinc ions in worts can be influenced by the elemental level present in the malt, the brewing process and modification of the malt (Jacobsen *et al.*, 1981). Processing parameters of the mashing cycle such as temperature, pH value, dilution of the mash and the time held at mashing temperature can effect wort zinc ion levels (Holzmann and Piendl, 1976). Elevated mashing temperatures result in precipitation of nitrogenous compounds removing chelating components which, in turn, remove ions from the wort. A considerable amount of zinc is lost during wort boiling and clarification operations (Jacobsen *et al.*, 1981; Daveloose, 1987). It is therefore apparent that malt composition and mashing conditions have a profound effect on zinc ion concentrations in brewer's wort.

Some studies have attempted to determine the minimum zinc ion concentration in wort that will allow yeast growth (Helin and Slaughter, 1977; Jones and Greenfield, 1984a)However a more recent study measured zinc ion concentration

within yeast in order to ascertain the bioavailability of this important ionic nutrient. It was found that nearly all of the zinc ions present in the wort were absorbed by the yeast in the first 20 h of the fermentation period. The maximal intracellular zinc ion concentration also occurred 20 h into the fermentation period, after which the intracellular concentration decreased due to the dilution effect of yeast cells dividing into daughter cells. Over seven repitches the intracellular zinc ion concentraion of a brewing yeast strain ranged from 17-26 μg (g dry wt)$^{-1}$ (Lentini *et al.*, 1990).

Magnesium ions are essential in fermentative metabolism where they participate in a number of enzymatic events. These include stimulation of synthesis of essential fatty acids, alleviation of inhibition by alkali earth metals, activation of ATP-ase (Suomolainen *et al.*, 1967) and a host of glycolytic enzymes, and regulation of cellular ionic levels (Rodríguez-Navarro and Sancho, 1979). In addition to these metabolic effects magnesium ions are important in maintaining membrane integrity (Diamond and Rose, 1970) and in control of cell growth and division in both budding and fission yeast (Walker and Duffus, 1980). Magnesium ions appear to have a greater involvement in protein-protein interactions than in lipid-lipid interactions (Jones and Greenfield, 1984a).The studies of Lentini *et al.* (1990) show that magnesium ions are absorbed from wort by yeast and a maximal intracellular concentration is reached after 40 h of the fermentation period. Over seven repitches the intracellular magnesium ion concentration of a brewing yeast strain ranged from 880-1370 μg (g dry wt)$^{-1}$ (Lentini *et al.*, 1990).

ETHANOL TOLERANCE IN YEAST

Production of alcoholic beverages such as beer, saké, whisky and wine have evolved over thousands of years and each type of beverage has spawned an industry. For many years it was generally accepted that the maximum concentration of ethanol produced in each of these beverages was a result of the intrinsic ability of the different yeast strains used. It was a widely held belief, therefore, that saké yeasts,

which produce up to 20% (v/v) ethanol, were more tolerant to ethanol than brewer's yeasts, which produce beers of 4-5% (v/v) ethanol. Traditionally, the order of ethanol tolerance amongst *Saccharomyces* species was saké yeast > wine yeast > distiller's yeast > brewer's yeast. Despite its importance little was known, until recently, about the physiological nature of ethanol tolerance in yeast. A major drawback in the area concerns the lack of an agreed definition of what ethanol tolerance is and how to assay it.

Defining and assaying ethanol tolerance

Although there is no agreed method for measuring ethanol tolerance in yeast there are, however, a number of means of assaying ethanol tolerance depending upon the chosen definition. Ethanol tolerance has been variously defined as, the ethanol concentration that will completely suppress batch growth under carefully defined conditions, the concentration of ethanol at which fermentative activity ceases, the highest concentration of ethanol produced by a yeast and the concentration of ethanol that causes a rapid loss of yeast viability.

The most widely used method is the first mentioned. Inoue *et al.* (1962) and Rose (1980 b) defined ethanol tolerance in *Saccharomyces cerevisiae* as the concentration of ethanol which just prevented yeast growth under defined conditions. Inoue *et al.* (1962) used saké yeast strains and found ethanol tolerances ranging from 11-12.5% (v/v) in medium containing 8% (w/v) glucose at 17°C. Rose (1980 b) used a defined glucose-salts medium containing 1% (v/v) increments of ethanol at 30°C. A brewing strain had considerably less tolerance, 7% (v/v) ethanol, than did three strains originating from distilleries and saké brewers, 12-13% (v/v) ethanol. Similar studies on commercial brewer's yeasts showed that ale yeasts were slightly less tolerant to ethanol in worts than lager yeasts. The previous history of the yeast, particularly its propagation (whether aerobic or anaerobic) and the nutritional composition of the wort, were found to influence its ethanol tolerance (White, 1978).

Ethanol tolerance has also been defined as the concentration of ethanol at

which fermentative activity ceases. This method assumes that substrate is non-limiting (Casey and Ingledew, 1985) and ethanol tolerances of 13 and 14.5% have been recorded for *Saccharomyces cerevisiae* PY-1 and UQM 73Y, respectively (Curtain *et al.*, 1984). The rate of viability loss has also been used as a means of assessing ethanol tolerance in a number of different yeast strains, (Day *et al.*, 1975; Inuoe *et al.*, 1962; Kalmakoff and Ingledew, 1985) as well as to measure the influences of nutritional and environmental conditions on ethanol tolerance (Brown *et al.*, 1981; Day *et al.*, 1975; Thomas *et al.*, 1978). Ethanol tolerance has been measured as the highest concentration of ethanol produced. In saké fermentations the reported high ethanol tolerances of yeast are measured in this way (Hayashida *et al.*, 1974, 1976). Saké fermentations differ from the batch fermentations of the brewing, distillery and wine industries, in that the substrate is added step-wise over a period of 30 days or more. In such a fermentation a saké yeast, *Saccharomyces cerevisiae* var.*saké* Kyokai no.7, was reported to tolerate 20% (v/v) ethanol (Hayashida *et al.*, 1974). Using a brewer's yeast, in a saké-type fermentation, Steinkraus *et al.* (1981) reported the production of 25.6% (v/v) ethanol.In batch fermentations, Kalmakoff and Ingledew (1985) showed a saké yeast to be less tolerant to ethanol, than ale, baker's and lager yeast strains.

Ethanol toxicity

Ethanol is the major product of yeast fermentations. Yet, at certain concentrations, ethanol is toxic to the yeast cell, as well as other microbes. Ethanol inhibits growth by denaturing proteins. For many years man has exploited this property of ethanol, using it as a sterilising agent. Given the antimicrobial activity of ethanol it is to be expected that the microbe most efficient at producing it, namely the yeast *Saccharomyces cerevisiae*, is more tolerant to its toxic effects than most microbes. However, it is widely believed that ethanol produced during the course of a fermentation is toxic to yeast cells.

Ethanol inhibits growth and fermentation by *Saccharomyces* species in a non-

competitve manner (Brown *et al.*, 1981). Thus ethanol affects the maximum specific rates for fermentation and growth, but not substrate affinity. Depending upon the experimental data obtained, growth inhibition rate can follow either a linear (Holzberg *et al.*, 1967; Roman *et al* .,1984), exponential (Jones and Greenfield, 1984 b; Louirero-Dias and Peinado, 1982; Leao and Van Uden, 1982), or hyperbolic model (Brown *et al.*, 1981; Novak *et al.*, 1981). More complex models for the rate of inhibition of growth have also been postulated (Brown *et al.*, 1981). These differences can be attributed to the wide variety of yeast strains, medium composition and growth conditions employed by different researchers (Mota *et al.*, 1984; Day et al., 1975; Thomas and Rose, 1979).

The range of ethanol concentrations produced in brewery, distillery, saké and wine fermentations is 4-20% (v/v). For many years it was presumed that the ethanol concentration within a yeast cell during fermentation was similar to that recorded in the external medium. However, various groups have reported intracellular accumulation of ethanol during the initial phase of fermentation. Intracellular accumulation of ethanol is believed to account for its toxic effect (Beavan *et al.*, 1982; D'Amore *et al.*, 1987, 1988; Loureiro and Ferrara, 1983; Nagodawithana and Steinkraus, 1976; Navarro and Durand, 1978; Novak *et al.*, 1981; Panchal and Stewart, 1980; Stewart *et al.*, 1988). There was general agreement that by the end of fermentation the difference between internal and external ethanol concentrations was small. However, other workers have shown that yeast do not accumulate ethanol at any point during fermentation (Dombek and Ingram, 1986 a; Guijarro and Lagaunas, 1984; Dasari *et al.*, 1990).It now seems clear that the reason for such confusion over this matter has arisen from the methodologies used to measure intracellular ethanol concentration. In addition to the methodology employed, it seems that recent reports of intracellular ethanol accumulation could be due to differences in yeast strain, and the difficulties associated with low cell densities and low extracellular ethanol concentrations at the beginning of batch fermentations (D'Amore *et al.*, 1988).

It is evident that the ethanol concentration found in the external environment approximates to that found in yeast cells. Since ethanol denatures proteins it is feasible that, at the concentrations found in yeast cells during fermentations, it exhibits its toxic effect by inhibiting enzymes and / or transport proteins. The studies of Millar *et al.* (1982) and Larue *et al.* (1984) suggest that inhibition of glycolytic enzymes does not play a major role in ethanol inhibition of yeast growth. Ethanol is known to inhibit the transport of glucose (Thomas and Rose, 1979; Leao and Van Uden, 1982), maltose (Louirero-Dias and Peinado, 1982), amino acids (Leao and Van Uden, 1984a) and ammonium ions (Leao and Van Uden, 1983) in *Saccharomyces cerevisiae*. Ethanol and other alkanols are known to disturb the membrane potential of yeasts (Leao and Van Uden, 1984b; Cartwright *et al.*, 1986). Ethanol increases proton influx thus dissappating the membrane potential and, as a consequence, disrupts active transport mechanisms. A recent report has cited accumulation of AMP, coupled with ethanol-induced disappation of the membrane potential, as a cause for inhibition of ethanol fermentation by yeast (Dombek and Ingram, 1988). Given that less than 20 proteins out of over two thousand have been studied, it is possible that ethanol may exhibit a specific toxic effect against a protein that has yet to be investigated.

Role of lipids and the plasma membrane in ethanol tolerance

The plasma membrane is the site controlling the transport of nutrients into the cell and the excretion of waste products into the surrounding medium. As early as 1948, ethanol tolerance in *Saccharomyces* species was known to be influenced by their lipid content (Gray, 1948). In order to understand the importance of the plasma membrane in yeast tolerance to ethanol it is necessary to briefly outline its composition. The plasma membrane of yeast contains phospholipids (Nurminen *et al.*, 1976) and free sterols (Nurminen *et al.*, 1975). The major yeast phospholipids are phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phophatidylserine and diphosphatidylglycerol (Ratledge and Evans, 1987). Phosphatidylcholine is the major phospholipid accounting for 25-55% of the total

followed by phosphatidylethanolamine. The most common fatty-acyl residues of phospholipids are $C_{16:0}$, $C_{16:1}$, $C_{18:0}$ and $C_{18:1}$. Polyunsaturated fatty-acyl residues do not occur in *Saccharomyces cerevisiae* due to the absence of the appropriate Δ^{12}-desaturase (Ratledge and Evans, 1987). The major role of the fatty-acyl residues of phospholipids would appear to be maintenance of the fluidity of the plasma membrane.

The principal yeast sterol is ergosterol followed by dehydroergosterol and lanosterol (Ratledge and Evans, 1987). Other sterols occur in minor quantities. In the plasma membrane, ergosterol is regarded as fulfilling a structural role (Nes, 1974; Nes and McKean, 1977). Grunwald-Raji and Margalith (1990) have recently confirmed ergosterol to be essential for the production of high levels of ethanol by yeast. They used nystatin-resistant mutants, some of which were defective in ergosterol synthesis, and which produced cholestatetraenol and zymosterol. Mutants that did not form ergosterol produced less ethanol, and had a lower viability, than parent strains during fermentations of glucose medium. A nystatin-resistant mutant with a capacity to synthesize ergosterol had a similar fermentation performance when compared with the parent strain.

Given the amphipathic nature of both plasma-membrane lipids and ethanol it is certain that such molecules interact directly with each other during fermentation to induce physiological changes to the membrane. Dombek and Ingram (1984) showed that ethanol increased the proportion of monounsaturated residues and the fluidity of the plasma membrane in *Escherichia coli*. It was postulated that the hydroxyl group of ethanol acts near the polar surface of the phospholipid bilayer, and the hydrocarbon portion penetrates the hydrophobic part of the bilayer, having a fluidizing effect. Electron spin resonance studies of membranes from *Saccharomyces cerevisiae* UQM 73Y and PY-1 have confirmed that ethanol acts primarily near the membrane surface, with ethanol-induced fluidization being confined to the hydrophobic part of the membrane nearest the polar head group (Curtain *et al.*, 1984). Such changes can influence the tolerance of yeasts to ethanol. Therefore, yeast membrane-lipid

composition, and the factors affecting it, are important in determining ethanol tolerance in yeasts.

There is evidence that ethanol tolerance of *Saccharomyces cerevisiae* can be enhanced following their acquisition of exogenous lipids. In saké fermentations, the presence of koji mould mycelia has been reported as essential for formation of high ethanol concentrations (Hongo *et al.*, 1967). It was later demonstrated that the essential component was a proteolipid fraction from *Aspergillus oryzae* (Hayashida *et al.*, 1974). The major phospholipid present in the proteolipid was phosphatidylcholine, the major fatty-acyl residue being linoleic acid. Small amounts of ergosterol were also found (Hayashida *et al.*, 1976; Hayashida and Ohta, 1978). It was found that the protein moiety could be replaced by albumin or methylcellulose without compromising production of high concentrations of ethanol (Hayashida *et al.*, 1976). When present at 1.5% (w/v) the proteolipid promoted production of 20.4% (v/v) ethanol compared with 17.1% (v/v) in its absence, by *Saccharomyces saké* Kyokai no. 7 (Hayashida *et al.*, 1974).

The proteolipid complex was shown to enhance "alcohol durability" of yeast during a 48 h soaking in 20% (v/v) ethanol, since protoplasts burst in its absence but remained intact in its presence (Hayashida *et al.*, 1975). When investigated further it was found that the proteolipid "strengthened" the protoplast membrane (Hayashida and Ohta, 1978). A complex of Tween 80®, ergosterol and albumin (Hayashida and Ohta, 1978) was able to replace the proteolipid and increase peak ethanol concentration from 17.2% to 19.0% in a 25-day saké fermentation with *Saccharomyces saké* Kyokai no. 7 (Ohta and Hayashida, 1983).

Oxygen, as discussed earlier, is required by brewer's yeast for the synthesis of sterols and unsaturated fatty acids (Andreasen and Stier, 1953, 1954). Such lipids are present in low concentrations in normal gravity brewing worts (David and Kirsop, 1972). In high-gravity worts, oxygen solubility is diminished as the sugar concentration increases. Since growth ceases once a limiting value of unsaturated lipids is reached (Kirsop, 1982), the low solubility of oxygen in high-gravity worts

increases the likelihood of growth related attenuation problems. Such problems with stuck fermentations have been reported (Day *et al.*, 1975).

Casey *et al.* (1983) found that the supplementation of wort (specific gravity 1.115) with 1% (w/v) yeast extract, 40 p.p.m. ergosterol and 0.4% (v/v) Tween 80® (an oleic acid source) had an influence on the fermentation performance of a commercial lager yeast. Fermentation time was decreased from two weeks, without supplements, to four days and 9.1% (v/v) ethanol was produced. It was found that the lipid component was more stimulative than the yeast extract, although both were required for maximum stimulation (Casey *et al.*, 1984). By increasing the pitching rate, it was possible to lower the level of supplementation. Further studies revealed that yeast cell mass and free amino-nitrogen utilization were greater in supplemented fermentations than in non-supplemented ones (Casey *et al.*, 1984). Since viability at the end of the fermentation in non-supplemented cultures was 98.9% of the peak viability during the fermentation, and the fermentative tolerance was not improved by the supplements, it was concluded that the sluggish fermentation of non-supplemented wort was due to a lack of nutrients. These nutrients were provided by the supplements. The lipid fraction of the supplements could be replaced by oxygen sparging (Casey *et al.*, 1984).

By exploiting the auxotrophic anaerobic growth requirement of yeast for a sterol and an unsaturated fatty acid, workers have been able to produce yeast cells rich (up to 70%) in a specific supplied sterol (Hossack and Rose, 1976) and in residues of a specific supplied unsaturated fatty-acid (up to 55%) (Thomas *et al.*, 1978). A series of studies with *Saccharomyces cerevisiae* NCYC 366 explored the influence of lipid membrane composition on ethanol tolerance. It was reported that membranes enriched in linoleyl residues ($C_{18:2}$), rather than oleyl residues ($C_{18:1}$), retained a greater cell viability in phosphate buffer containing 1 M ethanol. Moreover, this protection by unsaturated fatty-acyl residues was increased if the sterol had an unsaturated side chain (ergosterol or stigmasterol) rather than a saturated side chain (cholesterol or campesterol). Sterols with unsaturated side chains would have a

condensing effect on membrane phospholipids, decreasing membrane fluidity. It was proposed that sterols with unsaturated side chains increased cell viability by creating a more effective barrier to external ethanol (Thomas *et al.*, 1978).

Further studies with the same yeast (Thomas and Rose, 1979) revealed, that when anaerobically-grown exponential-phase cultures were exposed to 1.5 M ethanol, the increase in generation time was less in cultures enriched with ergosterol and linoleyl-residues (2.5 to 4.8 h gen^{-1}), than in cultures enriched with ergosterol and oleyl-residues (2.4 to 7.7 h gen^{-1}). In the same report, when suspensions of *Saccharomyces cerevisiae* NCYC 366 were exposed to 0.5 M ethanol, uptake of labelled glucose, arginine and lysine was inhibited to a lesser degree in cells with membranes enriched in linoleyl-residues rather than oleyl-residues. Thomas *et al.* (1978) proposed that ethanol decreased membrane fluidity by displacing water molecules at the headgroups of the phospholipids, decreasing repulsion between them and, as a result, decreasing fluidity. This was better compensated for by linoleyl residues than oleyl residues. However, more recent reports from different workers have shown ethanol to increase membrane fluidity (Curtain *et al.*, 1984; Jones and Greenfield, 1987 a; Walker-Caprioglio *et al.*, 1985).

Environmental influence on ethanol tolerance

High concentrations of substrate and ethanol are known to have a profound affect on the fermentative performance of yeasts (Hähn-Hagerdahl, 1982). Ethanol and substrate sugars can affect the ethanol tolerance of yeast by reducing the water activity (Jones and Greenfield, 1987 b). When yeast is inoculated into a medium it is exposed to osmotic pressure. If the concentration of wort solutes is less than that of the yeast solutes the yeast will absorb water and excrete some solutes until equilibrium is reached. If the concentration of yeast solutes is less than that of the wort solutes the yeast will lose water to the surrounding medium and will depolymerize some high molecular weight compounds to increase its internal osmotic pressure (Owades, 1981). The osmotic pressure increases during the fermentation of a

wort to a beer, for example, a wort of specific gravity 1.048 exerts an osmotic pressure of 790 Pa, whereas 80% apparent degree of fermentation of the same wort increases the osmotic pressure to 2.28 x 10^3 Pa (Owades, 1981). Increased glucose concentrations, above 15% (w/v), are known to decrease fermentation rate in yeast (D'Amore *et al.*, 1990; Jones *et al.*, 1981). Stewart *et al.* (1988) demonstrated that the inhibition was due to osmotic pressure and not catabolite repression. By using a base level of glucose (10% w/v) and substituting with sorbitol, a non-fermentable sugar, Stewart and his co-workers were able to alter the osmotic pressure of the medium while the glucose concentration remained unchanged. As osmotic pressure increased so fermentation rate, cell viability and ethanol production decreased (Stewart *et al.*, 1988). Yeast produces glycerol to counteract the rise in external osmotic pressure (D'Amore *et al.*, 1987). The glycerol acts as a "compatible solute" as the yeast adjusts its internal osmotic pressure to its external environment (Jones and Greenfield, 1987 b). Jones and Greenfield (1987 b) stated that solutes can inhibit fermentation in two ways; (1) reduce external water activity, a non-specific effect, and (2) via specific interactions against the cell.

EFFECTS OF CARBON DIOXIDE ON YEAST GROWTH AND FERMENTATION

It is well recognised that, as fermentation proceeds, so the efficiency of the process decreases. This is probably due to the build up and toxic effect of one of the major products of fermentation, namely ethanol (see previous section). However, little is heard of the inhibitory effects of the other major product of fermentation, carbon dioxide. Generally, low concentrations of carbon dioxide stimulate growth whereas high concentrations inhibit growth and metabolism (Slaughter, 1989).

Physico-chemical properties of carbon dioxide

To evaluate the inhibitory effect of carbon dioxide on yeast growth, one must first consider some of the physico-chemical properties of the gas in solution. The concentration of the various molecular species of carbon dioxide in liquid phase can be described according to the following association / dissociation relationships (Ishizaki *et al.*, 1971).

$$CO_{2\ (gas\ phase)} \leftrightarrow CO_{2\ (aqueous\ phase)}$$

$$CO_{2\ (aqueous\ phase)} \leftrightarrow H_2CO_3$$

$$H_2CO_3 \leftrightarrow HCO_3^-\ (bicarbonate\ ion) + H^+$$

$$HCO_3^-\ (bicarbonate\ ion) \leftrightarrow CO_3^{2-}\ (carbonate\ ion) + H^+$$

In typical yeast fermentations (pH 4.0-5.5), the major portion of the dissolved carbon dioxide is in the aqueous phase. The carbonate ion is effectively absent below pH 8. Carbon dioxide in fermentation broths behaves in a non-ideal manner, its solubility being a function of the concentration of other non-polar solutes such as ethanol and sugars (Jones and Greenfield, 1982). Ethanol and sugars decrease the solubility of carbon dioxide as does increasing temperature.

Effects of carbon dioxide on yeast growth

Carbon dioxide can stimulate yeast growth. In gas flow fermenters, where a partial pressure of carbon dioxide (pCO_2) values of less than 10^5 Pa can be maintained, it has been shown that pCO_2 values up to 3×10^4 Pa stimulates yeast growth (Slaughter, 1989). Under anaerobic conditions, carbon dioxide provided 6.5% of the total carbon of *Saccharomyces cerevisiae* grown on glucose in a chemostat (Oura *et al.*, 1980). Under the same conditions, with an excess of aspartate, only 1.6% of total carbon was fixed from carbon dioxide.

In contrast to the stimulative effect of carbon dioxide at low concentrations, the inhibitory effects at high concentrations are harder to explain. There are a number of possible mechanisms for carbon dioxide inhibition. These include a mass-action effect, effects on specific enzymes and membrane effects. The mass-action effect

attempts to explain carbon dioxide inhibition through direct product inhibition of the many metabolic decarboxylation reactions. However, there is little evidence to substantiate this theory since glycolysis, the major carbon dioxide-producing step, is not known to be affected by pCO_2 values of 4×10^5 Pa, whereas inhibition of growth occurs at pCO_2 values of 0.5×10^5 Pa (Slaughter, 1989).

Carbon dioxide can influence cellular membranes in a number of ways. Bicarbonate ions can affect the surface charge of membranes thereby influencing uptake of solutes such as amino acids, anions and cations which require an electrical potential to provide energy for their transport. Carbon dioxide $_{(\text{aqueous phase})}$ can react with amino groups to form carbamates (Mitz, 1979) at pH values above the isoelectric points of proteins. Carbamate formation can alter membrane protein conformation and thus alter membrane properties. Carbon dioxide $_{(\text{aqueous phase})}$ can also dissolve within the lipid matrix of the membrane rendering the membrane more fluid (Slaughter, 1989). The fatty-acyl composition of yeast exposed to carbon dioxide changes considerably with an increase in the proportion of unsaturated fatty-acyl residues and total lipid contents (Castelli *et al.*, 1969).

An excess carbon dioxide pressure of 2.9×10^5 Pa at 25°C prevented cell division in *Saccharomyces cerevisiae* (Norton and Krauss, 1972). That this effect was due to the gas and not pressure was proved by the demonstration that nitrogen used at the same excess pressure and temperature did not prevent cell division. Others have investigated this phenomenon in both budding and fission yeasts, and have produced essentially the same results (Lumsden *et al.*, 1987). It was found that carbon dioxide-induced changes could be reversed to some extent by releasing the carbon dioxide pressure.

Effects of carbon dioxide on fermentation by yeast

Fermentation of worts, by yeast, under carbon dioxide pressure has been used to speed up fermentation in production of lager beers. The traditional lager fermentation is long and slow and the easiest means of speeding it up, by increasing

the temperature, results in undesirable flavour compounds. However, the use of carbon dioxide at a pressure of 2 x 10^5 Pa increased the fermentation rate and did not affect the organoleptic properties of the beer (Slaughter, 1989). Problems with yeast viability have caused some breweries to end this practice. Kunkee and Ough (1966) attempted to use carbon dioxide pressure (2.5-5 x 10^5) to accelerate fermentation of a base wine to produce a sparkling wine. However, carbon dioxide was found to be inhibitory to yeast growth, especially at low pH values and high ethanol concentrations, as well as fermentation rate. By using yeast adapted to the base wine for several weeks, rapid fermentations were possible under carbon dioxide pressure.

It has been reported that gently agitated fermentations become supersaturated with carbon dioxide while vigorously agitated fermentations do not (Delente and Gurley, 1968). Others have demonstrated that vigorous shaking of supersaturated fermentation broths results in a sudden and voluminous release of carbon dioxide (Rice *et al.*, 1974). Supersaturation levels of carbon dioxide have been recorded in brewery fermentations (Trolle, 1950; Akin and Krabbe, 1966). Carbon dioxide is known to affect the uptake of amino acids by yeast in fermentations (Knatchbull and Slaughter, 1987; Slaughter *et al.*, 1987). Absorption of Group A amino acids (arginine, asparagine, aspartate, glutamate, glutamine, lysine, serine and threonine) was not affected whereas uptake of Group B amino acids (histidine, isoleucine, leucine, methionine and valine) was slowed down at carbon dioxide pressures of 0.5 x 10^5 and 1.01 x 10^5 Pa (Knatchbull and Slaughter, 1987). The concentration of amino acids in the medium decreased over the first four hours but then began to increase until, after 24 h, the concentration in the medium was higher than at the outset. Detailed investigation revealed, that during the initial four hours of fermentation, some amino acids were not absorbed at all, some were absorbed more rapidly, or more slowly, and some were not affected. It was postulated that the carbon dioxide-induced increase in cell volume could disrupt internal cellular organisation, releasing proteases from the vacuole thereby hydrolysing cell proteins. The increase in internal amino-acid concentration would inhibit uptake mechanisms allowing a net movement

of amino acids to the exterior (Slaughter *et al.*, 1987). Although this hypothesis has not been proven, it is obvious that carbon dioxide-supersaturation of media can have a profound affect on yeast fermentation performance.

PARTICULATE MATERIAL IN FERMENTATIONS

Trub is the name given to the insoluble material formed either during wort boiling or on cooling of the wort prior to fermentation. These two types of trub can be distinguished by the terms hot-break trub, formed during wort boiling, and cold break trub, formed during wort cooling. The hot-break trub is made up of insoluble tannin-protein complexes, insoluble salts, some hop resins, heavy metals and a proportion of the lipid material in the sweet wort and hops (Vernon, 1984). Most research suggests that the protein fraction is β-globulin and albumin from the malt which passes through the mashing process unchanged. The tannins of hot-break trub are of two types: those derived from the malt (anthocyanogen type) and those derived from hops (tannic type). Both types of tannin material play an active part in the formation of hot-break trub. Cold-break trub forms as wort cools and, like hot-break, is a protein-tannin complex. Protein accounts for 50-60% of hot-break trub, tannins, 20-30%, resins, 10-15%, and ash 2-3%. Cold-break trub is composed of protein, 50-70%, tannins, 20-30%, resins, 10-15% and ash 2-3% (Vernon, 1984).

Many factors affect break formation,including the liquor composition and its treatment, grist composition, malt quality, particularly its nitrogen content, and the mashing conditions used. Mashing at high temperatures brings more complex nitrogenous materials into solution, favouring trub formation. It has been shown that mashing under conditions that exclude oxygen produces worts and beers that resist clarification. The pH value also affects trub removal, with low pH values favouring aggregation and settlement of proteinaceous materials (Vernon, 1984). Production of bright worts has long been held desirable, but whether it is essential is a matter of some debate. Turbid worts, however, have been implicated in deterioration of beer

stability. If a break is defective a large proportion of sludge may appear in the fermentation vessel and may result in poor yeast performance.

Wort clarification

Wort can be clarified by either sedimentation or filtration. In filtration systems, solid particles are removed by virtue of their size by a screen placed in the liquid flow. In sedimentation systems, solids are removed by virtue of their density difference from the surrounding medium. To achieve optimum hot-break trub removal, clarification must take place at the highest possible temperature where the difference in particle and wort density is at its greatest and wort viscosity is lowered.

Hop backs have been used in many breweries for trub removal. The hop back is a vessel similar to a mash tun and is fitted with a slotted base. Wort from the copper is circulated from under the plates on to the top of a hop filter bed. Hot-break trub is deposited on the hops on the compacted bed. The bed depth is critical and is usually about 0.3 m. Many breweries have had problems in obtaining a proper bed formation and depth. Brewers also believe the wort quality to be poor due to standing wort on the hops.

Other systems for wort clarification include filtration and centrifugation. Filtration of hot wort through plates and frame filters is practised in some European breweries. Centrifugal clarifiers have been in use for many years and produce a relatively clear wort. However, the remaining sludge needs cleaning out and the operation is non-continuous. Centrifuges have been developed to eject solids thus making their use continuous. However, such centrifuges are costly.

A cheaper method of trub removal, the whirlpool tank, was developed by Molson Breweries in the 1960's. The whirlpool tank is usually a cylindrical vessel into which wort is pumped at high velocity. The wort entering the tank rotates around the vessel's vertical axis. In the upper layers of the liquid this throws particles outwards due to centrifugal forces. When the tank is full, the wort at the bottom of the tank moves more slowly than the wort above, because of frictional forces between the

vessel base and the liquid. A pressure gradient builds up which causes wort to flow inwards at the vessel bottom and outwards at the top. This circulatory pattern within the liquid volume throws trub particles into the centre of the vessel where they are deposited as a cone. A number of factors appear critical for efficient whirlpool function. These include, tank shape, the ideal height-to-diameter ratio being 0.6 : 1, the location of the wort inlet, ideally 0.5-1 m above vessel base at a tangent 5-30° to the vertical, and the speed at which wort is pumped into the tank. A rotational stand of about 30 min seems to be adequate for effective trub removal. Clarified wort is drawn off to fermentation vessels via an outlet and trub is removed by spraying with water.

Effects of particulate materials on fermentation

Reports of increased yeast growth rate and acceleration of fermentation by residual insoluble material in distiller's malt-wort (Merritt, 1967) and increased yeast activity, fusel alcohol and glycerol production in fermentations with trub solids present (Hough, *et al.*, 1982) suggest that solid particles play an important role in fermentations. However, suspended solids are implicated in yeast autolysis and depressed head formation in the final beer (Merritt, 1967). Merritt (1967) studied the effect of wort solids on fermentation of distiller's wort and found that they stimulated fermentation regardless of the state of aeration of the wort, suggesting that the effect was not due to entrainment of oxygen in the particles. Wort solids were found to have a positive effect on yeast growth in amino nitrogen-limited cultures, whereas no such effect was observed in carbohydrate-limited cultures. It was hypothesized that a combination of electrostatic and absorptive forces concentrate yeast cells and amino acids at the solid-liquid interface, thereby increasing the rate of uptake of amino acids by the cell, and diverting carbohydrate to yeast growth (Merritt, 1967).

More recently, trub has been shown to stimulate yeast activity and fermentation rate, decrease ester synthesis and increase production of fusel alcohols (Schisler *et al.*, 1982). In their initial experiments, Schisler and his co-workers compared yeast performance in fermentations of partially filtered, normally aerated

worts with that of yeast in normally aerated, high-trub worts. Attenuation was more rapid and the suspended yeast count greater in fermentations of high-trub worts. Further investigations showed that high-trub, low-oxygen worts fermented at the same rate as filtered, high-oxygen worts and faster than clarified worts. Clarified worts had lower lipid and zinc-ion contents than high-trub worts. Since unsaturated fatty acids from spent grains had successfully stimulated yeast growth in deoxygenated worts (Taylor *et al.*, 1979), and zinc is an important metal in biosynthetic pathways, it was concluded that the stimulative effect of trub was related to its high lipid and zinc-ion contents (Schisler *et al.*, 1982). However, no evidence was furnished for greater uptake of lipids or zinc-ions by yeast from fermentations of high-trub worts; it was only inferred. Indeed evidence suggested that most of the zinc-ions in the high-trub wort were bound up in the trub.

A different interpretation of the effect of trub solids on yeast performance in brewery fermentations was put forward by Siebert *et al.* (1986). They found that addition of the lipidic fraction of trub caused a slight stimulation of fermentation. However, effects similar to that of trub, i.e. a rapid drop in specific gravity, increased yeast crop yields and viability, were observed when other solid particles, such as activated carbon or diatomaceous earth, were added to clarified worts. It was proposed that the particles serve as nucleation sites for carbon dioxide bubble formation. Altering the size of the activated carbon particles did not significantly affect yeast fermentation performance. The possibility that activated carbon absorbed inhibitory substances, such as medium-chain fatty acids, was dismissed since diatomaceous earth, though not as absorbent as activated carbon, produced a similar effect when added to fermentations of clarified wort. Carbon dioxide levels in fermentations with solids added were 30-35% lower than those in controls. This is significant when one considers that reports suggest that carbon dioxide levels may reach one and a half times saturation level in commercial fermentations (Rice *et al.*, 1977). Addition of solids would bring carbon dioxide levels back to saturation point. The effects of trub in promoting vigorous fermentation, increased yeast growth and

higher viability appeared to be associated with the physical nature of the particle as a solid rather than with any nutritional effect (Siebert *et al.*, 1986).

To understand the principles behind nucletion in brewery fermentations it is necessary to examine what is meant by the term. Interest in nucleation stems from the desire of chemical engineers to predict the boiling characteristics of any combination of solid heater and fluid coolant. The onset of nucleate boiling can be brought about by either heterogeneous or homogeneous nucleation. Only the former type of nucleation need be considered when addressing the phenomenon of carbon dioxide evolution in brewery fermentations. In heterogeneous nucleation boiling is initiated by growth of bubble nuclei from trapped gas pockets in the many cavaties on a heater surface. When initial bubble nucleation is considered the system is in equilibrium hence the onset of nucleate boiling (ONB) concerns only the physical properties of the fluid and its interaction with the solid (Shock, 1982). Equilibrium of a bubble requires an excess of pressure inside it given by:

$$\Delta p = \frac{2\sigma}{R}$$

where Δp = excess pressure , σ = surface tension and R = radius of the bubble. A bubble in s surface cavity takes the form of a spherical segment of radius R_e, hence

$$\Delta p = \frac{2\sigma}{R}$$

Assuming dp_v / dT is independent of p over the range Δp, the superheat requirerment for bubble formation is given by

$$T_{sat\ ONB} = \frac{2\sigma}{R_e\,(dp_v / dT)}$$

where σ, R_e and dp_v / dT may be complex functions of the physical porperties and composition of the mixture. The onset of nucleate boiling can be affected by the presence of dissolved gases in the system, for this case eqution (1) above can be rewritten,

$$p_v + p_i - p_l = \frac{2\sigma}{R}$$

where p_v = vapour pressure, p_i = partial pressure of the gas and p_l = liquid pressure. Hence a significant contribution from the gas to the total pressure in the bubble can

reduce the necessary vapour pressure and thus the superheat requirement for nucleus stability. Although the equations would be more complex the same principles apply to nucleation of dissolved carbon dioxide in yeast fermentations.

Solid particles are reported as having effects similar to those of trub in wine fermentations. Ough and Groat (1978) demonstrated increased fermentation rate and yeast flocculation, as compared to controls, in fermentations of white grape juice supplemented with grape solids. More recently, the addition of yeast ghosts to stuck fermentations of wine must has been shown to have a beneficial effect (Munoz and Ingledew, 1989 a, b; Lafon-Lafourcade *et al.*, 1984; Ribéreau-Gayon, 1985). Solid particles, namely γ-alumina granules, have been demonstrated to stimulate ethanol production in rapid industrial fermentations (Kanellaki *et al.*, 1989).

Given the contradictory nature and paucity of literature reports regarding the stimulative effect of trub, and other solids, in fermentations further investigations are required to elucidate the factor(s) responsible.

AIMS OF THE PROJECT

The aim of this study was to investigate aspects of the anaerobic growth of *Saccharomyces cerevisiae*. In the first section the effect of particulate material (trub) on the fermentation performance of yeast was studied. Of special interest in this investigation were those components of particulate material that are known to have an advantageous affect on yeast fermentation performance, namely sterols, unsaturated fatty acids and trace metals.

METHODS

ORGANISM

The yeast (a strain of *Saccharomyces cerevisiae*) used in this study was a top-cropping ale yeast provided by Whitbread and Company. Cultures of the yeast were maintained at 4°C on slopes containing (l^{-1}) : malt extract (lab m) 3.0 g; yeast extract (lab m) 3.0 g; glucose 10 g; peptone (lab m) 5.0 g and agar 20 g (MYGP; Wickerham, 1951).

EXPERIMENTAL FERMENTATIONS

Brewer's wort and glucose-salts medium fermentations were carried out in fermentation tubes (height 300 mm; diameter 30 mm). These tubes were fitted with two sampling ports sealed with suba seal bungs. Figure 2 shows the system used for laboratory fermentations.

Glucose-salts medium, similar to that used in anaerobic uptake experiments, was used in addition to brewer's wort for fermentations. The glucose-salts medium contained (l^{-1}) : glucose 150 g; $(NH_4)_2SO_4$ 3.0 g; KH_2PO_4 4.5 g; yeast extract (lab m) 1.0 g; $CaCl_2.2H_2O$ 25 mg; $MgCl_2.7H_2O$ 25 mg. The pH was adjusted to 4.5 with concentrated HCl. A glucose concentration of 150 g l^{-1} yielded a medium of specific gravity 1.060. Media of different specific gravities were prepared by altering the glucose concentration. A 200 ml portion of medium was dispensed into each fermentation tube. Where indicated media were supplemented with untreated or treated trub. Tubes containing media were sterilised by autoclaving at 10lb in^{-2} ($6.89x10^4$ Pa) for 1 min. After autoclaving the tubes were clamped upright and incubated at 30°C. When cool, each portion of medium was inoculated with 400 mg wet weight of an 18 h starter culture of the yeast. Starter cultures (100 ml of glucose-salts medium; sp.gr.1.060) were inoculated with a pinhead of organisms from a slant

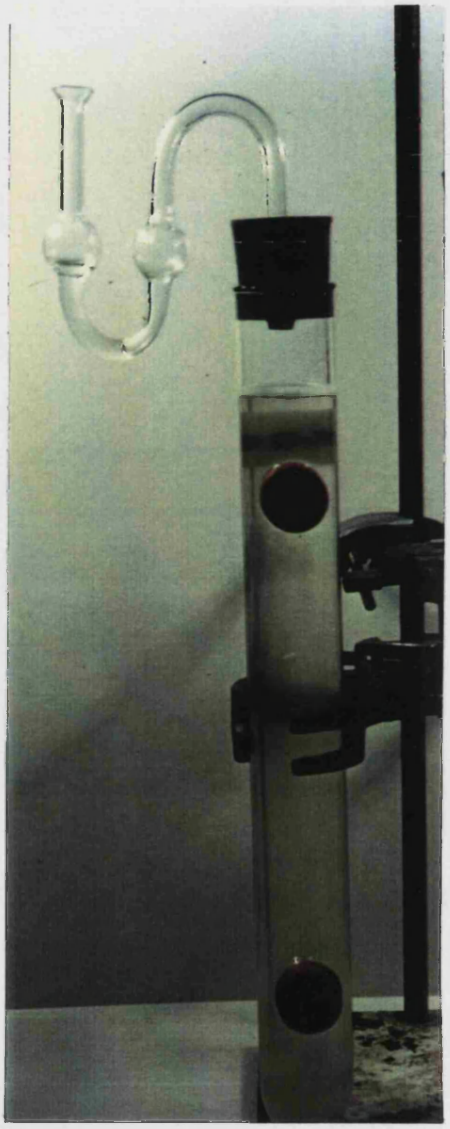


FIGURE 2. Fermentation tubes used for laboratory fermentations showing stoppered injection ports and the fermentation lock.

culture and were incubated at 30°C for 18 h on an orbital shaker (200 r.p.m.). Starter culture viability was checked and greater than 85% viability was deemed acceptable. In some experiments maltose-salts medium, in which maltose replaced glucose, was used.

Wort samples, from brews of Whitbread Best Bitter were obtained from Whitbread and Company's Cheltenham Brewery, were clarified by centrifugation (4000 *g*; 10 min; 4°C) and adjusted to the required specific gravity by addition of distilled water. A 200 ml portion of clarified wort was dispensed into each fermentation tube. Where indicated tubes were supplemented as described previously. Fermentation tubes containing brewer's wort were steam-sterilised at 100°C for 5 min.The tubes were then incubated and inoculated as already described.

COLLECTION OF TRUB

Hot-break trub was collected from the whirlpool at the Cheltenham Brewery of Whitbread and Company after wort for Whitbread Best Bitter brews had been drawn off to the fermentation vessel. The whirlpool was accessed via a hatch and hot-break trub samples obtained using a steel cup attached to a wooden pole. Both cup and pole were swabbed with 70% (v/v) methanol prior to use. Samples were placed into sterile waste-disposal bags, refrigerated at 4°C for 1 h, and transported back to Bath in an ice-box.

Cold-break trub was obtained from cooled wort by centrifugation. Wort was sampled from a sampling point on the "cold" side of the paraflow heat exchanger. A sterile sampling kit consisting of two steel needles connected by clear plastic tubing was used to pierce a self-sealing membrane and a sterile sampling bottle. Methanol (70%, v/v) was swabbed over the membrane and the sampling bottle to minimize microbial activity. Six litres of wort were collected, centrifuged (1200 *g*; 10 min), and the cold-break obtained pooled as a slurry. Replicates of both hot- and cold-break trub samples were distinguished from each other by their brewery gyle number.

On arrival at Bath hot-break samples were centrifuged (4000 *g*; 10 min; 4°C) and washed three times with distilled water (4000 *g*; 10 min; 4°C). Samples were then frozen at -20°C overnight prior to freeze-drying (Centrifugal Freeze-Dryer Model 30P2/727; Edwards High Vacuum Ltd., Crawley, England). Cold-break samples were centrifuged (4000 *g*; 10 min; 4°C) to deposit the solid material and the pellet washed three times with distilled water (4000 *g*; 10 min; 4°C). Samples were frozen and freeze-dried as described above.

Wort samples were collected as described for cold-break trub collection, refrigerated at 4°C, and transported back to Bath in an ice box.

FRACTIONATION OF TRUB

Lipid extraction

Lipid was extracted from trub by a modification of the method of Hunter and Rose (1972). Ethanol (80%,v/v; 80 ml) at 80°C was added to 2 g of freeze-dried trub and the suspension maintained at that temperature, with occasional stirring, for 15 min. The extract was filtered through Gelman Science GN-6 Grid 45 μm filters and the trub scraped off the filter with a spatula into a flat-bottomed flask containing chloroform-methanol (2:1,v/v; 120 ml). The suspension was stirred on a flat-bed magnetic stirrer at room temperature for 3 h, filtered, and the filtrate added to the ethanol extract. The residual trub was re-extracted with chloroform-methanol (2:1,v/v; 120 ml) for 3 h, then for 2 h, and the extracts combined. The lipid-extracted trub was scraped into a china crucible and dried *in vacuo* for 4 h.

The combined lipid extract was transferred into a separating funnel, washed with 25% volume 0.88%(w/v) KCl (Folch *et al.*, 1957), and left to seperate overnight at -20°C. The lower organic layer was collected and evaporated to dryness using a Büchl-Rotavapor EL rotary evaporator (Büchl, Switzerland) and an electrothermal water bath (Electrothermal, England) set at 40°C. The lipid residue was resuspended

in 1 ml of hot ethanol (100%,v/v; 80°C) and injected into sterile medium using a sterile syringe and needle.

Water extraction

The water-soluble fraction of trub was extracted by washing 1 g of trub in 30 ml distilled water for 3 h at room temperature. The suspension was filtered through Gelman Science GN-6 Grid 45 μm filters, and the filtrate and insoluble trub were collected. The filtrate was made up to 200 ml with distilled water and supplemented with the appropriate components to give a medium with a specific gravity of 1.060. This medium was dispensed into tubes and autoclaved as already described. The water-insoluble residue was dried for 8 h in a vacuum oven at 60°C.

Ashing

Ashed trub was prepared by placing 1 g of trub into an acid-washed fused silica crucible (VitreoSil, England) and heating in a electric muffle furnace (Carbolite, Sheffield, England) at 900°C for 18 h or until a grey-white ash was obtained. Ashed trub was washed for 30 min in 10 ml sterile deionised water in a flat-bottomed flask on a flat-bed magnetic stirrer at room temperature. The suspension was filtered through Whatman no.541 ashless filter paper into a sterile universal and added using a syringe to media after autoclaving.

Cation depletion

The cation content, especially that of zinc, in trub was lowered by washing 2 g of trub overnight in 200 ml of $5x10^{-3}$ M EDTA at pH 5.5. The samples were magnetically stirred. Treated samples were filtered through Gelman Sciences GN-6 Grid filters, washed three times with deionised water and the trub residue scraped into a china crucible, and dried overnight at 60°C in a vacuum oven.

Lipid-extracted cation depleted trub was prepared by washing lipid-extracted

trub, described earlier, with EDTA as described above. Dried treated trub was ground and added to fermentation medium.

ANALYTICAL METHODS

Specific Gravity

The original and specific gravity of a medium, fermenting medium or wort were measured using a glass hydrometer (Boots). Hydrometer readings were made by removing the rubber stopper and placing the hydrometer into the fermentation tube. Precautions were taken to minimise the contamination risk: (1) the fermentation was allowed to establish itself before readings were taken; (2) the hydrometer was stored in 80% (v/v) ethanol to ensure sterilty; (3) the relatively small surface area : volume ratio decreased the risk of contamination and of oxygen entering the medium; (4) the inoculum and cell viabilty at the time of inoculation were sufficiently high to prevent competition from all but the most gross contamination.

Carbohydrate

Trub carbohydrate was estimated using the H_2SO_4-carbazole method (McMurrough and Rose, 1967). A solution of ground trub (2 mg ml^{-1}) was prepared in water. The solution was boiled for 5 min and the supernatant was analysed for carbohydrate. Duplicate portions (0.5 ml, diluted if necessary) of suspensions containing approximately 200 µg of trub extract were transferred to boiling tubes and aqueous H_2SO_4 (5.0 ml; 8 H_2SO_4:1 H_2O v/v) was carefully added to each tube. The tubes were vortexed (Whirlymix; 10 s) and allowed to cool at room temperature. Carbazole reagent (0.3 ml, containing 0.5% w/v carbazole in 95% v/v ethanol) was added to each tube. The tubes were covered with aluminium caps, vortexed (Whirlymix; 10 s), and the tubes placed in a boiling water bath for 10 min. After cooling the absorbance was measured at 535 nm in an LKB Ultrospec 4050 spectrophotometer. A glucose standard curve was also prepared.

Ethanol

Ethanol contents of fermented glucose-salts medium and wort were determined by GLC. Samples (1.5 ml) were withdrawn from the culture and transferred to Eppendorf tubes. Yeast cells were pelleted by centrifugation (MSE MicroCentaur, 11500 *g*) and the samples stored at -20°C. On defrosting, the supernatant was diluted 1 in 50 with water, mixed, and 0.5 ml added to an equal volume of 0.2% (v/v) propan-1-ol in water. The sample was mixed and 1 µl was injected onto the column of a PYE Unicam GCD gas chromatograph fitted with FID. The glass column (1.5 m long; 0.4 cm internal diameter) was packed with Chromosorb 101 (100/120 mesh) and maintained at 150°C. The injection temperature was 250°C and the nitrogen carrier gas flow rate was 40 ml min^{-1}. Ethanol concentration was determined by reference to the propan-1-ol internal standard. Standard solutions of 0.2% (v/v) ethanol and 0.2% (v/v) propan-1-ol were used to calculate response factors. Calculation of the ethanol concentration was aided by use of an LDC/Milton Roy CI-10B coumputing integrator.

Lipids

Yeast lipids

Washed yeast was frozen at -20°C overnight in preparation for freeze-drying. Lipids were extracted from freeze-dried cells by a modification of the method of Letters (1968). Ethanol (80%,v/v; 20 ml), containing 2.5 mg cholestanol and 2.5 mg heptadecanoic acid, at 80°C was added to portions (200 mg) of freeze-dried yeast cells and the suspension maintained at that temperature, with occasional stirring, for 15 min. The extract was cooled and filtered through Whatman no.44 filter paper and the residue further extracted with 30 ml of a 2:1 (v/v) chloroform-methanol (Bligh and Dyer, 1959; Folch *et al.*,1957) mixture stirred magnetically at room temperature for 2 h. The suspension was filtered, the filtrate pooled with the ethanol extract, and stored at -20°C. The residue was extracted a further two times for 2 h and 1 h, respectively, in 2:1 (v/v) chloroform-methanol (30 ml) and the extracts pooled as

already described. Pooled extracts were washed with 25% volume 0.88% (w/v) KCl (Folch *et al.*, 1957) and the mixture left to separate overnight at -20°C. The lower organic phase was removed, taken to dryness using a rotary evaporator and the residue dissolved in hexane (67-70°C).

The concentrated lipid extract was transferred to a Quickfit tube and evaporated to dryness using a Buchler Rotary Evapo-Mix (Buchler Instruments, New Jersey, U.S.A.). Lipids were saponified by modification of the method of Safe (1974). A freshly prepared solution of 2 M KOH in 95% (v/v) methanol (4 ml) and benzene (1 ml) was added to the tube, the lipid residue dissolved, and the suspension transferred to a clean dry bijou bottle. The headspace of the bijou bottle was flushed with nitrogen gas, the bottle capped, and heated in a Techne heating block at 80°C for 3 h. An equal volume of methanol was added and the non-saponified fraction extracted with 3x5 ml washes of hexane (67-70°C). The extracts were pooled in a Quickfit tube and taken to dryness using the Rotary Evapo-Mix. The residue was dissolved in 200 µl *bis*-(trimethylsilyl)trifluoroacetamide (BSTFA) in pyridine, transferred to a bijou bottle capped with an open top screw cap fitted with a Teflon laminated rubber disc (Alltech), and heated at 80°C for 15 min under nitrogen gas. The sterol content was analysed by GLC on a 25 m OV101 WCOT quartz capillary column in a PYE Unicam 4500 chromatograph (injector temperature 250°C; detector temperature 250°C). The initial column temperature was 200°C which was raised to 300°C at 4°C min^{-1} over 25 min . The helium carrier gas flow rate was 1 ml min^{-1} and the nitrogen make-up gas flow rate was 40 ml min^{-1} . Sterol peaks were identified by co-chromatographing with sterol standards . Response factors were calculated for each sterol relative to cholestanol and quantities of sterol determined. Calculations were aided by the use of a Trivector Trio computing integrator.

The remaining solution, containing the saponified lipids, was acidified using 1 ml 6 M HCl and the lipid extracted with 3x5 ml washes of chloroform. Lipid samples were transferred to Quickfit tubes and taken to dryness as already described The residue was dissolved in 1 ml 14% (w/v) boron trifluoride in methanol, and

transferred to a bijou bottle under nitrogen gas. The bottle was capped with an open top screw cap, fitted with a Teflon laminated rubber disc, and heated at 80°C for 1 h in a Techne heating block. The contents of the bijou bottle were allowed to cool and were tipped into a 10 ml graduated Quickfit test tube containing 4 ml distilled water. The suspension was washed with 3x5 ml chloroform to extract the fatty-acid methyl esters and the extract concentrated to a small volume by rotary evaporation. Fatty-acid methyl ester analysis was carried out on a 25 m SGE fused silica BPX70 capillary column in a PYE Unicam GCD chromatograph in conjunction with a Trivector Trio integrator. The sample was injected (1 *ul*) onto an on-column injection system (injector temperature 250°C; detector temperature 250°C). The column temperature was initially 135°C, held at that temperature for 2 min, then raised at 8°C min^{-1} until the final column temperature, 200°C, was reached and held for 2 min. The carrier gas was hydrogen. Fatty-acid composition was calculated by reference to the heptadecanoic acid internal standard using the Trivector integrator.

Trub lipids

Freeze-dried trub, prepared as already described, was ground with a pestle and mortar and 250 mg added to ethanol(80%,v/v; 20 ml), containing 2.5 mg cholestanol and 2.5 mg heptadecanoic acid as internal standards. The suspension was heated to 80°C and maintained at that temperature, with occasional stirring, for 15 min. The ethanol extract was cooled and lipid extracted as already described except that the lipid residue was dissolved in 10 ml hexane (67-70°C) instead of 1 ml. A portion (5 ml) of the lipid extract was evaporated to dryness using a Buchler Rotary Evapo-Mix and the lipid saponified, extracted and analysed as described for yeast lipid analysis. To analyse free fatty acids and sterols a 5 ml portion of the lipid mixture was evaporated to a small volume using a rotary evaporator. Using a 50 µl syringe, the sample was carefully streaked onto a 20x20 cm 0.25 mm thick Silica Gel G precoated TLC plate (Whatman). A mixture of lipid standards was streaked onto one side of the plate. After allowing time for the plate to dry, it was developed in a solvent mixture

consisting of light petroleum ether (40-60°C)-diethyl ether-acetic acid (70:30:1 by vol.) until the solvent front was 1 cm from the top edge. The plate was sprayed with 2',7'-dichlorofluorescein in ethanol and viewed under UV radiation (254 nm). Free fatty acid and free sterol bands were identified, marked, and scraped off into separate bijoux bottles. One ml of 14% (w/v) boron trifluoride in methanol was added to the free fatty acid extract. Free sterols were eluted with 3x5 ml washes with hexane (67-70°C). The free sterol fraction was taken to dryness and dissolved in 200 µl BSTFA. Both fatty-acid methyl esters and silylated sterols were prepared, extracted and analysed as already described.

To determine the total dry weight of trub lipid a portion (250 mg) of freeze-dried trub was added to ethanol (80%,v/v; 20 ml) at 80°C and held at that temperature, with occasional stirring, for 15 min. Lipid extraction proceeded as already described for total trub lipid analysis. When saponified and non-saponified lipid fractions were obtained they were transferred to clean, dry, pre-weighed bijoux bottles and dried to a constant weight.

Magnesium and zinc

Yeast cells were analysed for their magnesium and zinc content by atomic absorption spectrophotometry. Cells were prepared for analysis by a variation of the analytical methods recommended by the Ministry of Agriculture Fisheries and Food (MAFF, 1986). A 200 mg portion of freeze-dried yeast cells was ground to pass a 1 mm sieve and transferred to fused silica crucibles (all crucibles and glassware used were acid-washed). The crucibles were placed in a muffle furnace, the temperature increased to 800°C, and maintained overnight until a grey-white ash was obtained. The crucibles were removed from the furnace when sufficiently cool and covered with a watch glass. Hydrochloric acid (6 M; 5 ml) was added to the crucible, taking care that material was not lost due to effervescence. The crucible was placed on a hot plate, at a low heat, in fume cupboard and evaporated to dryness. Heating was continued for 1 h after the solution had evaporated. The residue was moistened with

2 ml HCl (approx 36%; Spectrosol, BDH, Poole, England), the crucible covered with a watch glass, and the contents gently boiled for 2 min. Deionised water (10 ml) was added and the solution boiled again. The watch glass was removed, washed with water and the washings collected in the crucible. The contents of the crucible were quantitatively transferred into a 25 ml volumetric flask and diluted to 25 ml. Removal of the remaining solid material was effected by filtering the solution through Whatman no.541 filter paper into a glass bottle.

The concentrations of magnesium and zinc were determined by atomic absortion on a SP 9 PYE Unicam atomic absorption spectrophotometer (Cambridge, England). Calibration curves for both elements were constructed using 0-1.0 μg (magnesium or zinc ml)$^{-1}$ solutions prepared from 1000 mg (magnesium or zinc l^{-1}) standards (Spectrosol). To eliminate interference from phosphate 10% (v/v) releasing reagent (60 g Spectrosol $SrCl_2.6H_2O$, dissolved in 800 ml deionised water, to which was added 100 ml of Spectrosol $HClO_4$ [approx 60% $HClO_4$] diluted to 1 l) was added to magnesium calibration and sample solutions. Magnesium sample solutions were diluted 1:100 prior to analysis. Zinc sample solutions were analysed undiluted. Magnesium was analysed at a wavelength of 285.2 nm using an air-acetylene flame (gas flow rate 1.0 l min^{-1}; lamp 4 mA; bandpass 0.5 nm). Zinc was analysed at a wavelength of 213.9 nm with gas conditons as for magnesium (lamp 7mA; bandpass 0.5 nm). Magnesium and zinc concentrations were determined by reference to their respective calibration curves and converted to μg (g freeze-dried yeast)$^{-1}$.

Freeze-dried trub was prepared for magnesium and zinc analysis by ashing 200 mg trub overnight in a muffle furnace at 800°C. Ashed trub was prepared for and analysed by atomic absorption spectrophotometry as already described. Trub treated with EDTA was analysed for minerals as described for yeast.

Magnesium and zinc analysis of glucose-salts medium and wort was carried out by freezing a portion (50 ml) of the medium at -20°C overnight. The frozen medium was then freeze-dried in centrifugal freeze-dryer (Edwards). It was then transferred to a fused silica crucible and ashed at 500°C overnight in a muffle furnace.

Ashed medium was prepared for and analysed by atomic absorption spectrophotometry as already described. Element concentrations were determined from their respective calibration curves and converted to μg (medium l)$^{-1}$.

Protein

The protein content of trub was assayed using the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany), a method based on the dye-binding technique of Bradford (1976). Samples (20 mg) of trub were boiled for 5 min with 1 ml 0.1 M NaOH. The suspension was then neutralised by addition of an equal volume of 0.1 M HCl. Portions of the solution, diluted if necessary, were mixed with 5 ml Bio-Rad dye reagent (20% v/v in water), vortexed (Whirlymix; 10 s) and the colour allowed to develop for 5 min at room temperature. The absorbance of the solution was then measured at 595 nm in a LKB 4050 Ultrospec spectrophotometer.
A calibration curve using bovine serum albumin was also prepared.

Tannin

Tannin content of trub was estimated by the revised vanillin assay (Price *et al.*,1978). Ground trub (200 mg) was added to 10 ml methanolic HCl (1% concentrated HCl in methanol), heated to 70°C, and maintained at that temperature, with occasional stirring, for 20 min. Assays were performed on the supernatant at 30°C with reagents previously warmed at that temperature. Vanillin reagent was prepared by mixing equal volumes of 1% vanillin in methanol and 8% concentrated HCl in methanol. Vanillin reagent was freshly prepared for each determination. Vanillin reagent (5 ml) was added to 1ml portions of the sample. A blank, in which 5 ml of 4% concentrated HCl in methanol was added to a 1 ml portion of sample, was prepared for each sample. After 20 min the absorbance was read at 500 nm on a LKB Ultrospec 4050 spectrophotometer and the absorbance of the blank subtracted from the test with vanillin. A calibration curve was constructed using catechin as a standard.

pH and suspended yeast count

The pH value of each fermentation was recorded using a PYE Unicam PW 9418 pH meter. The pH probe was first been dipped into 80% (v/v) ethanol to sterilise it and then dipped into fermenting medium. Samples for suspended cell counts were withdrawn from fermenting medium at the lower sampling port in Figure 2 using a sterile syringe. A Neubauer counting chamber was used for counting cells.

Yeast dry weight

Yeast dry weight was determined by drying the total solid matter obtained from a fermentation or starter culture to a constant weight in a cold vacuum oven. Yeast dry weight was obtained by subtracting the weight due to trub solids (if any were present) from the total dry weight.

ETHANOL TOLERANCE OF YEAST

Ethanol tolerance of yeast was defined as the highest concentration of added ethanol which allowed growth in 1.060 specific gravity glucose-salts medium after 24 h. Glucose-salts medium (specific gravity 1.060°) was autoclaved as already described. Ethanol was added to cool, sterilised, batches of medium to give final ethanol concentrations in the range 0-12% (v/v) in increments of 2% (v/v). Media (200 μl) were dispensed into sterile microtitre plates (Sterillin, Feltham, Middlesex, England) such that columns corresponded to the same ethanol concentration and rows a stepwise increase in ethanol concentration. A control column using distilled water was prepared. Media were inoculated with washed resuspended yeast (8 µl equivalent to [2 g wet weight l]$^{-1}$), sealed with Titretek plate sealer (Flow Laboratories, Rickmansworth, England), and incubated at 30°C with rotary shaking (200 r.p.m.). Growth was ascertained by measuring the optical density at 600 nm against the control blank using a MR 600 Dynatech microplate reader.

YEAST VIABLITY

The methylene blue counting technique used was based on the EBC Analytica method (1962). Sodium citrate dihydrate (2 g) was added to 10 ml of 0.1% (w/v) aqueous methylene blue solution and made up to 100 ml with water. An equal volume of this solution was added to an equal volume of yeast suspension. Over 300 cells were counted for each determination in an improved Neubauer counting chamber. Cells that stained blue were counted as dead and those unstained considered viable.

MATERIALS

All chemicals used were AnalaR grade or of the highest purity available. Trub and wort samples were obtained from Whitbread and Company, Cheltenham, England. Boron trifluoride, BSTFA and lipid standards were obtained from Sigma Chemical Co. Ltd., Poole, England. Atomic absorption spectrophotometry standards were obtained from BDH Co. Ltd., Poole, England. Gas-liquid chromotography columns were provided by Scientifc Glass Engineering Ltd., Milton Keynes, England.

RESULTS

TRUB COMPOSITON

Protein was the major component of hot-break trub. Ash, lipid and tannin were secondary components of the trub, with carbohydrate a minor component (Figure 3).

Trub lipid

The total saponified fatty-acyl and free fatty acid composition of cold- and hot-break trub varied considerably between the different gyles from the same brew (Tables 1,2,3 and 4). Predominant amongst the free fatty acids in both cold- and hot-break were $C_{16:0}$ and $C_{18:2}$, with $C_{18:1}$ the next most abundant (Tables 2 and 4). These residues were also the most common in total saponified fatty-acyl analyses of cold- and hot-break trub (Tables 1 and 3).

A component that co-chromatographed with β-sitosterol was the only sterol observed in the free sterol and total saponifed sterol fractions from hot-break trub. Values for β-sitosterol content varied widely between gyles. The total saponified β-sitosterol content in hot-break trub from gyles 80, 294 and 662 was 1.5 mg, 4.1 mg and 0.5 mg cholestanol equivalent $(g\ trub)^{-1}$ respectively. The total free β-sitosterol in hot-break from gyles 80, 294 and 662 was 1.3, 3.1 and 0.3 mg cholestanol equivalent $(g\ trub)^{-1}$ repectively. Trace amounts of β-sitosterol were found in both the free and total sterol fractions of cold-break. Lipid-extracted trub was found to contain 0.3 mg fatty-acyl residues $(g\ trub)^{-1}$ of which $C_{16:0}$ was the most abundant with 0.075 mg $(g\ trub)^{-1}$. Over 95% of the lipid was extracted from the trub.

Magnesium and zinc in trub

Trub contained 12.1 ± 0.6 mg magnesium $(g\ trub)^{-1}$ and 115.6 ± 13.7 μg zinc $(g\ trub)^{-1}$. The values were the mean of six determinations plus or minus the standard deviation. EDTA treated trub contained 13.9 ± 1.2 μg zinc $(g\ trub)^{-1}$. The values were

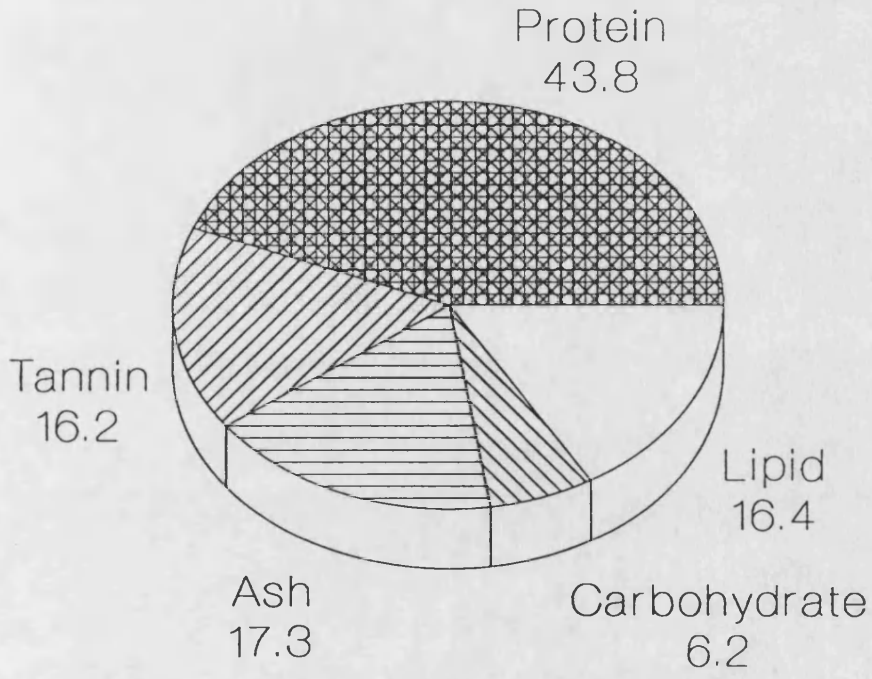


FIGURE 3. Pie chart showing trub composition as percentage by weight. Values quoted are the average of at least three independent determinations and the variation never exceeded the 10% confidence limit.

TABLE 1. The fatty-acyl composition of saponified lipid extracted from hot-break trub from three separate brews of Whitbread Best Bitter. The values are the average of three determinations (nd denotes not detected).

Component	amount present in mg $C_{17:0}$ equivalent (g trub)$^{-1}$		
Fatty-acyl residue	gyle 80	gyle 294	gyle662
$C_{14:0}$	nd	2.1 ± 0.15	nd
$C_{16:0}$	9.42 ± 1.09	19.1 ± 1.05	3.45 ± 0.25
$C_{16:1}$	0.72 ± 0.17	nd	nd
$C_{18:0}$	1.64 ± 0.29	1.11 ± 0.03	0.37 ± 0.07
$C_{18:1}$	4.58 ± 0.37	1.47 ± 0.12	0.92 ± 0.12
$C_{18:2}$	16.32 ± 0.92	3.53 ± 0.15	3.27 ± 0.47
$C_{18:3}$	3.40 ± 0.56	1.24 ± 0.10	0.49 ± 0.02

TABLE 2. The free fatty acid composition of lipid extracted from hot-break trub from three separate brews of Whitbread Best Bitter. The values are the average of three determinations (nd denotes not detected).

Component	amount present in mg $C_{17:0}$ equivalent (g trub)$^{-1}$		
Fatty acid	gyle 80	gyle 294	gyle 662
$C_{14:0}$	0.16 ± 0.06	0.45 ± 0.11	nd
$C_{16:0}$	3.94 ± 0.56	4.79 ± 0.64	3.33 ± 0.61
$C_{16:1}$	nd	3.34 ± 0.31	trace
$C_{18:0}$	0.15 ± 0.03	0.15 ± 0.02	0.15 ± 0.04
$C_{18:1}$	0.39 ± 0.04	0.68 ± 0.05	0.27 ± 0.09
$C_{18:2}$	1.64 ± 0.21	3.19 ± 0.61	1.44 ± 0.12
$C_{18:3}$	0.27 ± 0.08	0.55 ± 0.12	0.15 ± 0.07

TABLE 3. The fatty-acyl composition of saponified lipid extracted from cold-break trub from three separate brews of Whitbread Best Bitter. The values are the average of three determinations (nd denotes not detected).

Component	amount present in mg $C_{17:0}$ equivalent (g trub)$^{-1}$		
Fatty-acyl residue	gyle 80	gyle 294	gyle 662
$C_{16:0}$	3.68 ± 0.15	2.27 ± 0.36	0.52 ± 0.12
$C_{16:1}$	nd	nd	trace
$C_{18:0}$	1.03 ± 0.02	0.87 ± 0.11	0.14 ± 0.06
$C_{18:1}$	3.85 ± 0.28	0.71 ± 0.05	0.39 ± 0.10
$C_{18:2}$	6.30 ± 0.32	1.11 ± 0.15	0.33 ± 0.07
$C_{18:3}$	1.13 ± 0.09	nd	nd

TABLE 4. The free fatty acid composition in lipid extracted from cold-break trub from three separate brews of Whitbread Best Bitter. The values are the average of three determinations (nd denotes not detected).

Component	amount present in mg $C_{17:0}$ equivalent (g trub)$^{-1}$		
Fatty acid	gyle 80	gyle 294	gyle 662
$C_{16:0}$	0.65 ± 0.08	0.51 ± 0.02	0.48 ± 0.09
$C_{16:1}$	nd	nd	0.18 ± 0.04
$C_{18:0}$	nd	0.15 ± 0.03	0.1 ± 0.02
$C_{18:1}$	trace	0.18 ± 0.02	0.25 ± 0.05
$C_{18:2}$	0.29 ± 0.07	0.22 ± 0.05	0.1 ± 0.01
$C_{18:3}$	nd	nd	0.24 ± 0.05

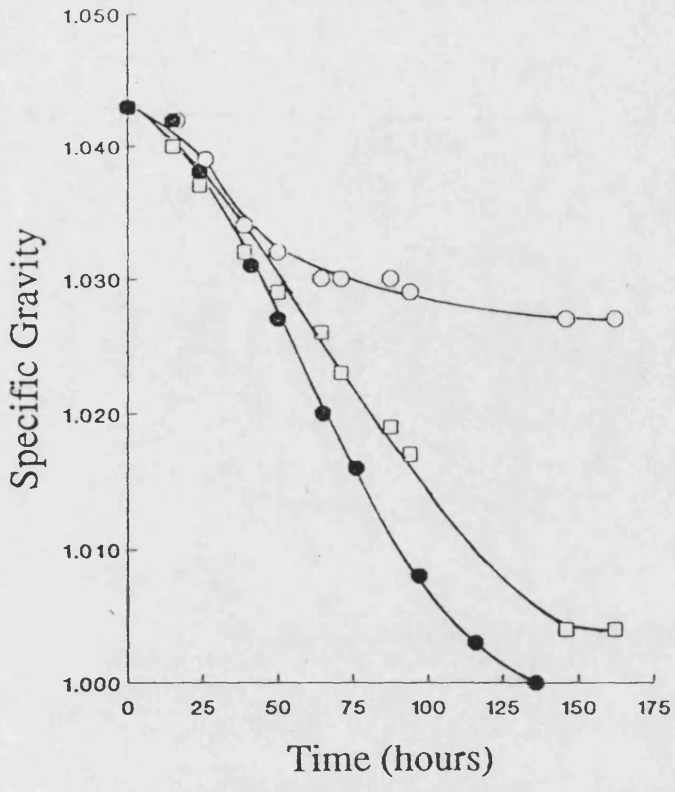
the average of three independent determinations plus or minus the standard deviation.

FERMENTATION OF GLUCOSE-SALTS AND MALTOSE-SALTS MEDIA

Effect of trub

Fermentations of glucose-salts media (original gravity 1.043) by *Saccharomyces cerevisiae* were stimulated in the presence of trub (Figures 4 (a),(b) and (c)). This was shown by manifestly quicker attenuation times in fermentations containing trub. The ability of trub from different gyles of the same grist to stimulate fermentations demonstrated that the effect could be replicated between gyles and was not gyle-dependent. The control fermentations stuck in one of the experimental series (Figure 4 (a)) but attenuated in the other two albeit at a slower rate than when 1 g trub was present (Figures 4 (b) and (c)). In one series of fermentations the presence of 250 mg trub in glucose-salts medium was found to be stimulative (Figure 4 (a)). However, in the other two series of fermentations, although the rate of attenuation over the first half of the fermentation was more rapid in the presence of 250 mg trub compared with the control, the total time taken to attenuate the medium was not significantly different (Figures 4 (b) and (c)). The stimulative effect of trub on the fermentation of glucose-salts medium by *Sacch. cerevisiae* became more apparent when the specific gravity of the medium was raised to 1.050 , 1.060 and 1.080 , respectively (Figures 5 and 6) In fermentations of glucose-salts medium of original gravity 1.050 the control fermentations attenuated but at a slower rate than those fermentations to which 1 g trub was added (Figure 5). However, with fermentations of glucose-salts media of original gravities 1.060 and 1.080 only fermentations in which trub was present attenuated while the control fermentations stuck.

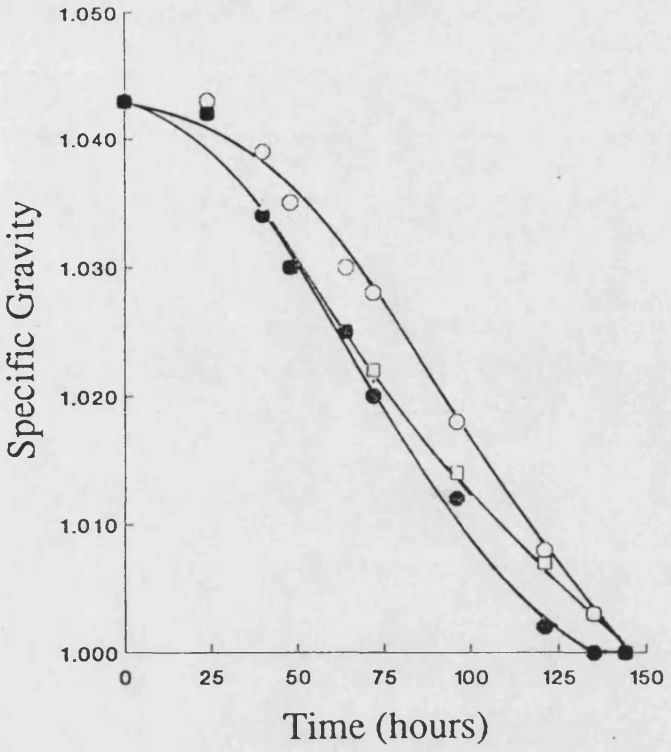
Fermentations of maltose-salts medium (original gravity 1.060) by *Sacch. cerevisiae* in the presence and absence of trub showed characteristics different from those of glucose-salts medium at the same specific gravity (Figure 7). There was no significant difference in the time taken to attenuate the medium between



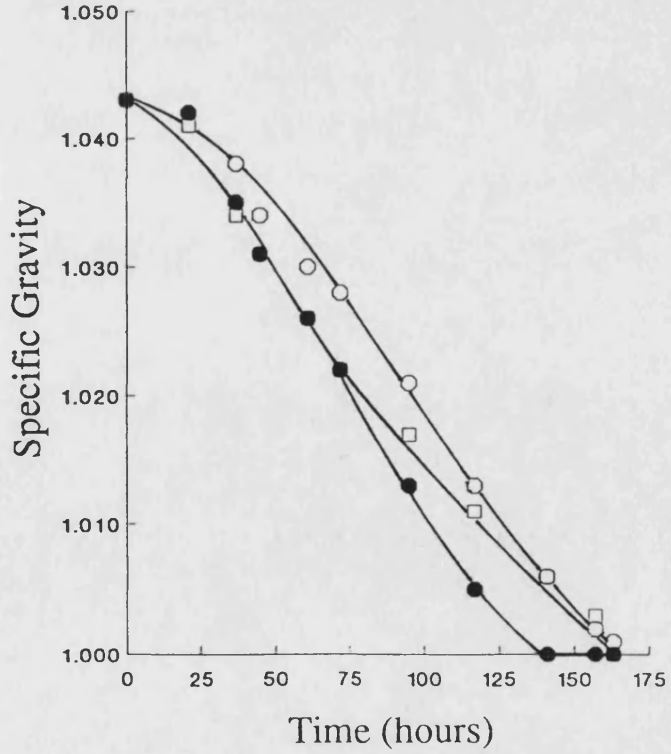
(a)

FIGURE 4. Time-course of fermentation of glucose-salts medium (original gravity 1.043°) by *Saccharomyces cerevisiae* showing the effect of trub collected from separate gyles of the same grist. Figure 4(a) shows the effect of trub from gyle 294; Figure 4(b) shows the effect of trub from gyle 80; Figure 4(c) shows the effect of trub from gyle 662. Grist composition, mashing and wort-boiling conditions were the same for each gyle. The values plotted were the average of three independent fermentations and the variation never exceeded the 10% confidence limit.

Key: (○) no trub control; (□) 250 mg trub; (●) 1 g trub.


1.050
1.040
1.030
1.020
1.010
1.000
Specific Gravity
0
25
50
75
100
125
150
Time (hours)

(b)


1.050
1.040
1.030
1.020
1.010
1.000
Specific Gravity
0
25
50
75
100
125
150
175
Time (hours)

(c)

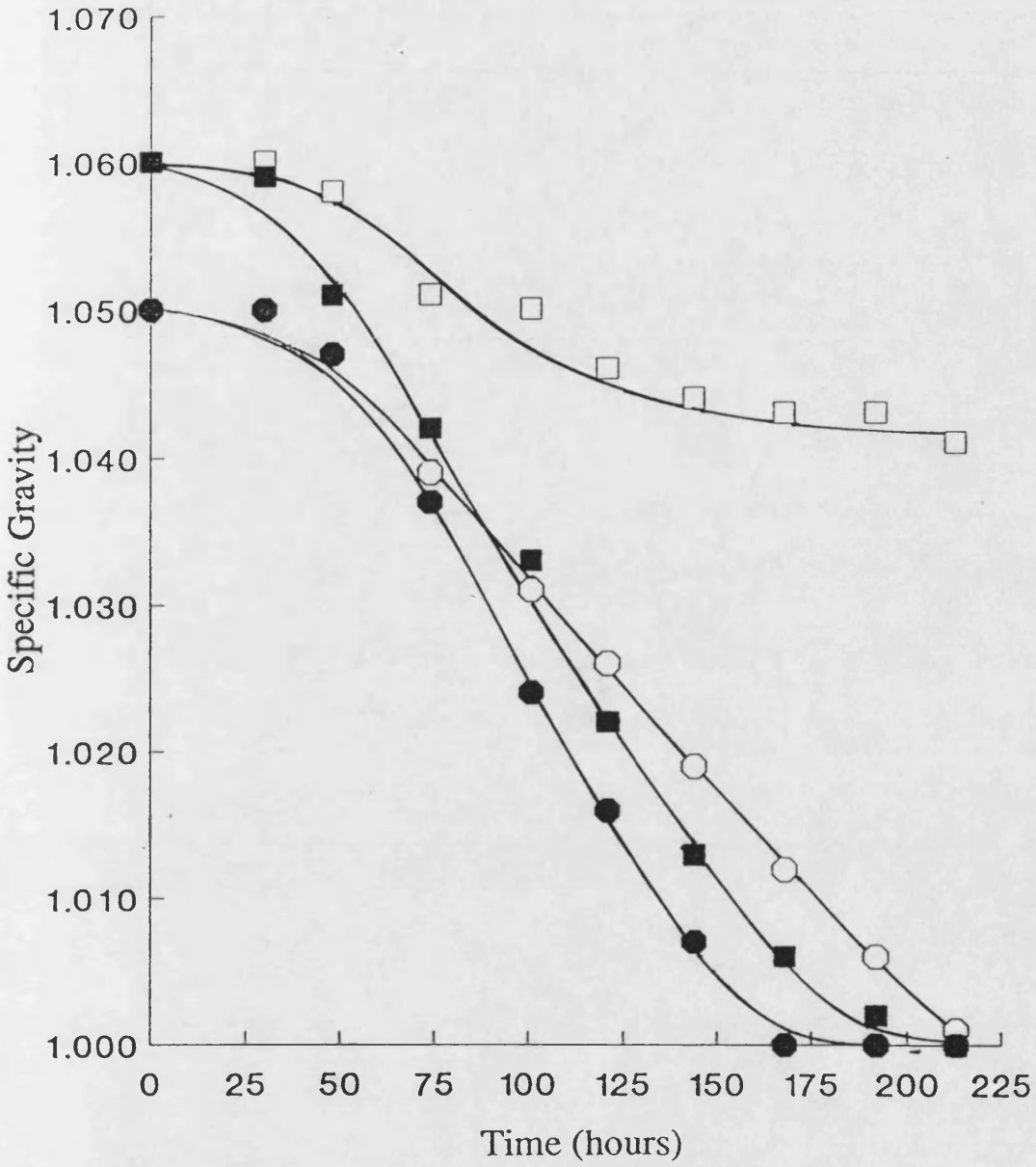


FIGURE 5. Time-course of fermentation of glucose-salts media (original gravities 1.050 and 1.060 , respectively) by *Saccharomyces cerevisiae* showing the effect of trub. The points were the average of three independent fermentations and the variation never exceeded the 10% confidence limit. Key (○) no trub control (1.050 original gravity); (●) 1 g trub (1.050 original gravity);(□) no trub control (1.060 original gravity); (■) 1 g trub (1.060 original gravity)

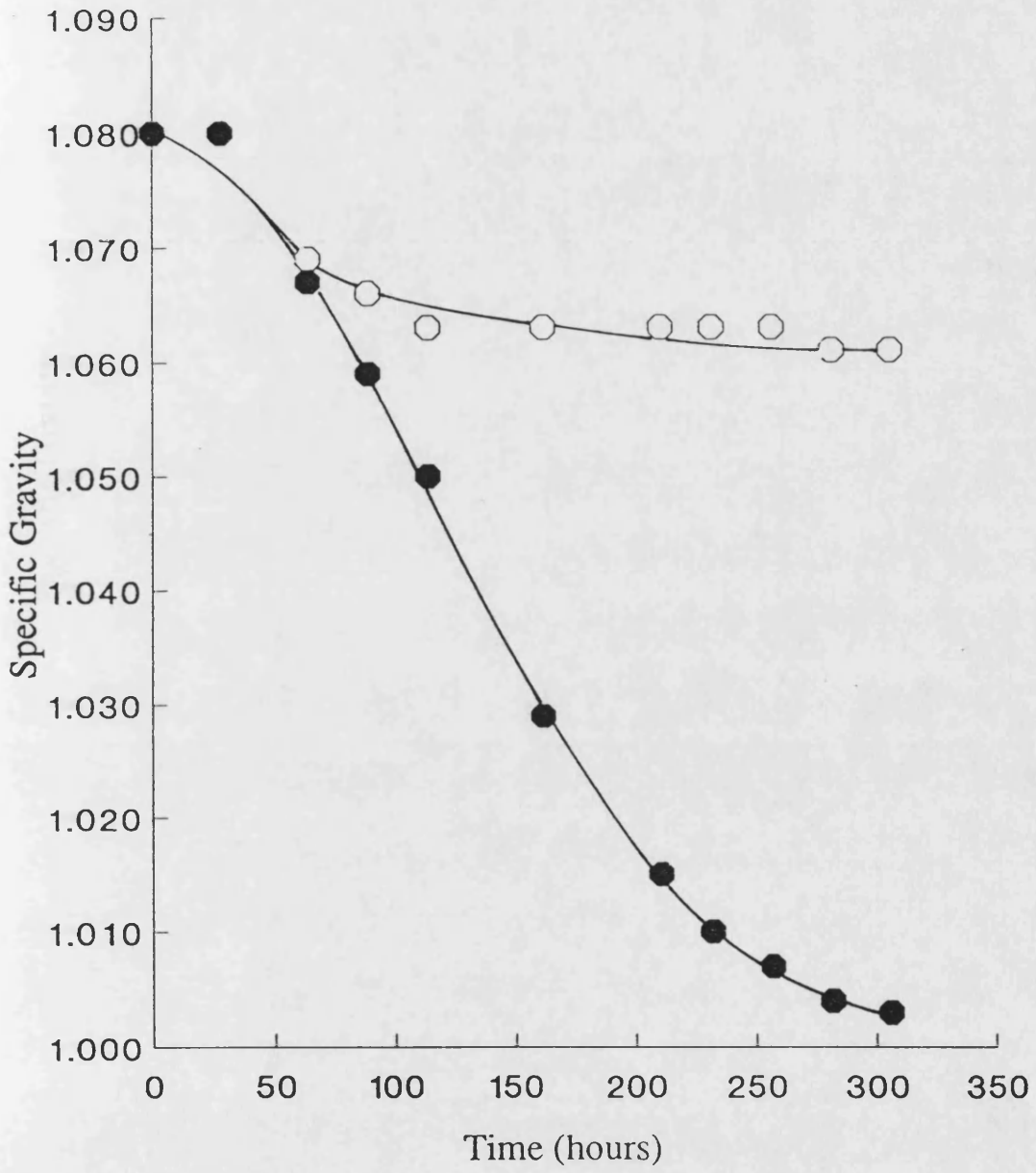


FIGURE 6. Time-course of fermentation of glucose-salts medium (original gravity 1.080) by *Saccharomyces cerevisiae* showing the effect of trub. The points were the average of three independent fermentations and the variation never exceeded the 10% confidence limit. Key (○) no trub control; (●) 1 g trub.

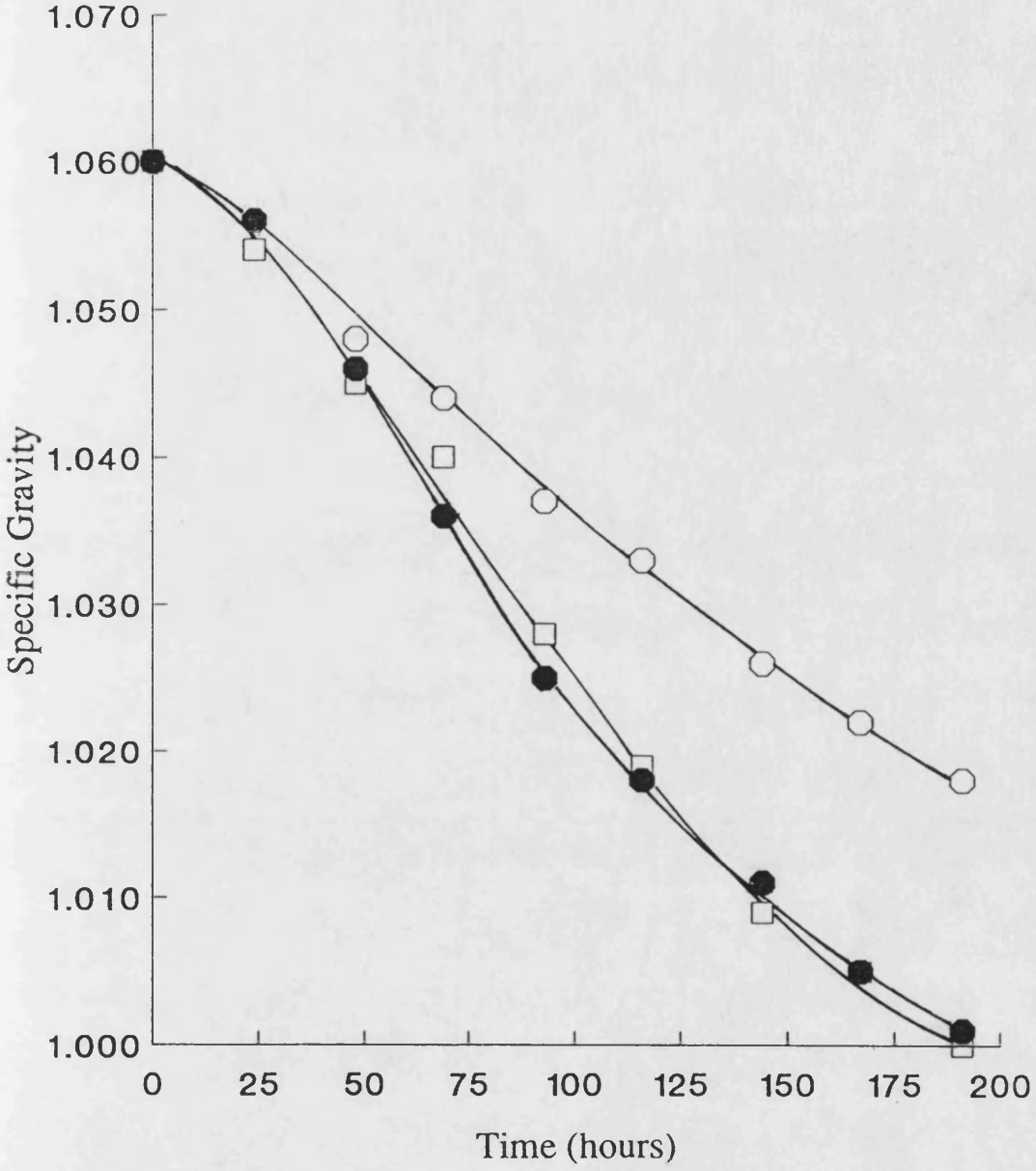


FIGURE 7. Time-course of fermentation of maltose-salts medium (original gravity 1.060) by *Saccharomyces cerevisiae* showing the effect of trub. The points were the average of three independent fermentations and the variation never exceeded the 10% confidence limit. Key (○) no trub control; (●) 250 mg trub; (□) 1 g trub.

fermentations to which 1 g or 250 mg trub had been added. Although the control fermentations were not stuck they had not attenuated the medium in the time that the trub-containing fermentations had.

The addition of 1 g sterilised trub to stuck fermentations of glucose-salts medium (original gravity 1.060) fermentations enabled the yeast to restart the fermentation although the medium was not fully attenuated (Figure 8). Trub was steam sterilised for 1 min prior to addition to the fermentation. Fresh trub, which was collected as already described in the Methods except that the freeze-drying step was omitted, was less effective than freeze-dried trub in stimulating fermentation of glucose-salts medium (original gravity 1.060) (Figure 9). The greater the amount of fresh trub added the greater was the stimulative effect on the fermentation. Generally fermentations to which trub was added attenuated more rapidly than the control fermentations. Yeast activity was greater in trub-containing fermentations than in control fermentations (Figure 10). Trub-containing fermentations showed greater CO_2 evolution, as indicated by foaming in the headspace, and more yeast in suspension, indicated by a greater degree of turbidity, than control fermentations.

Effect of trub fractions

Fractions of trub showed a range of capacities to stimulate the fermentation of glucose-salts medium (original gravity 1.060) by *Sacch. cerevisiae*. The water-insoluble fraction of trub displayed the greatest stimulative effect but was marginally less effective than whole trub (Figure 11). The water soluble fraction of trub induced greater attenuation of the glucose-salts medium than the control but the fermentation was sluggish and eventually stuck at a specific gravity of 1.025 . Lipid-extracted trub attenuated glucose-salts medium (original gravity 1.060) to a specific gravity of 1.010 after 270 h. Whole trub, however, fully attenuated the same medium after 170 h (Figure 12). The lipid extract from trub was initially inhibitory to fermentation although it enabled the yeast to attenuate the medium to a greater degree than the control fermentation. Fermentations to which trub lipid extracts were added

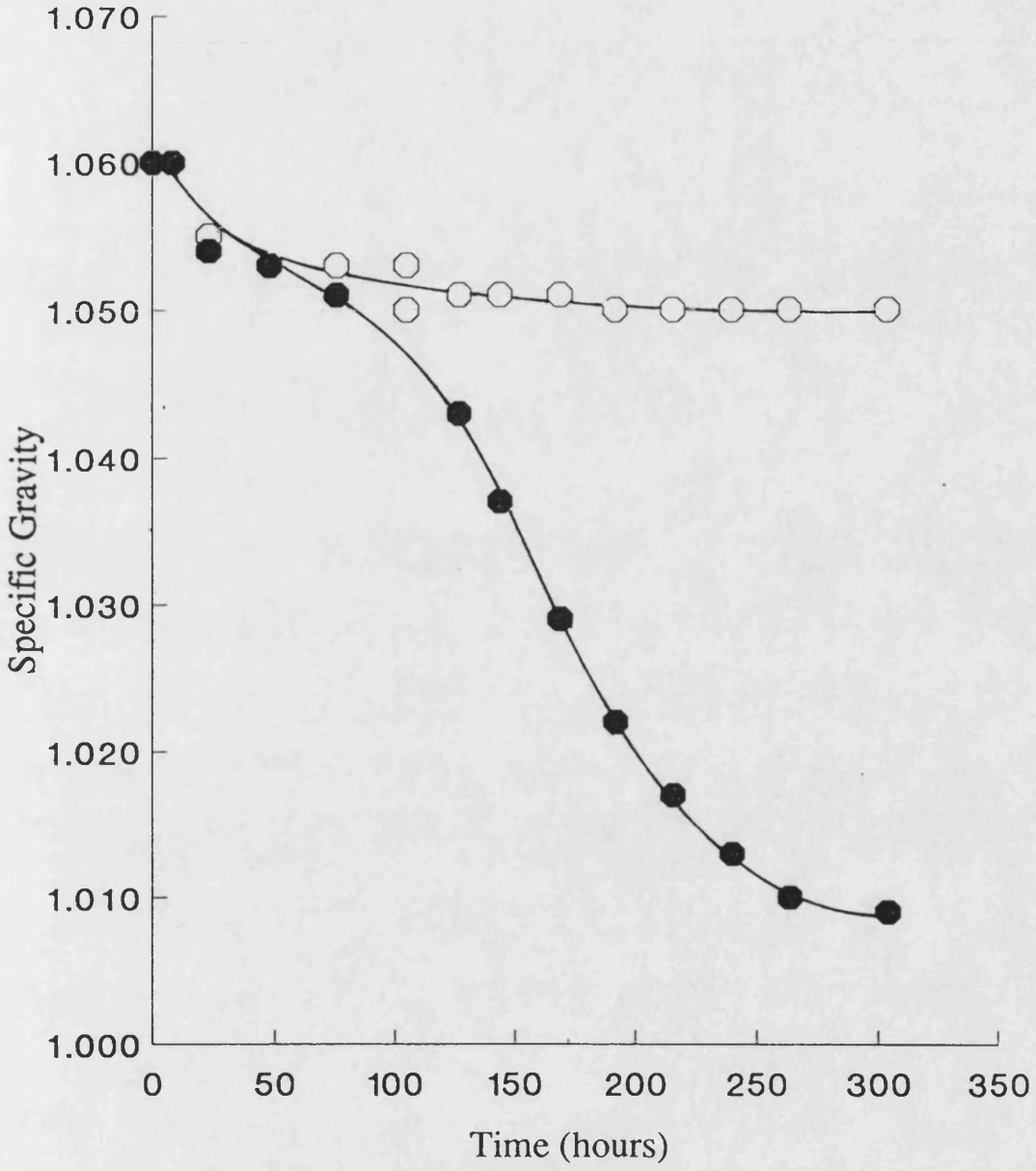


FIGURE 8. Time-course of fermentation of glucose-salts medium (original gravity 1.060) by *Saccharomyces cerevisiae* showing the effect of adding trub to a stuck fermentation. The points were the average of three independent fermentations and the variation never exceeded the 10% confidence limit. Key (○) no trub control; (●) 1 g trub added after 78 h.

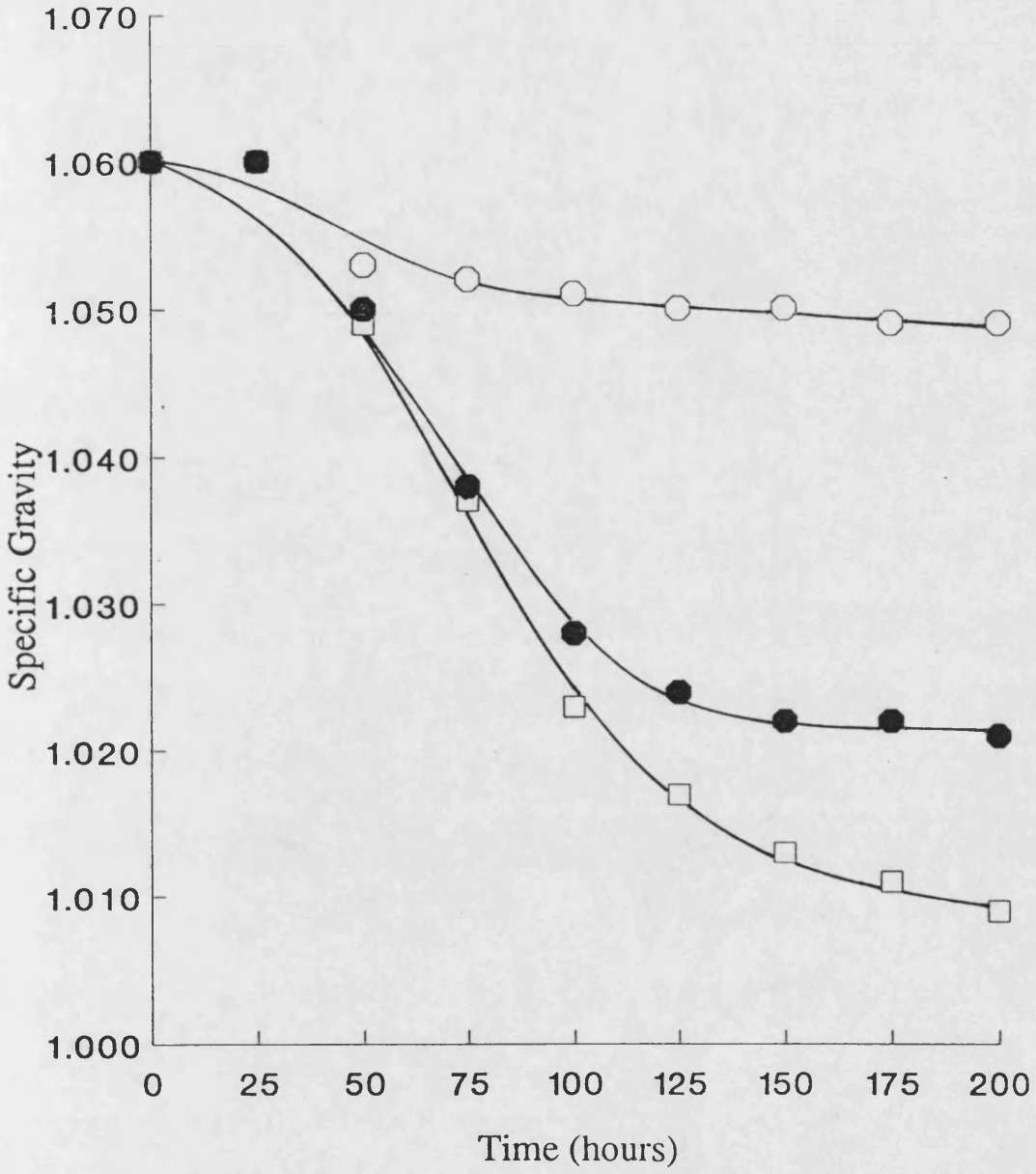


FIGURE 9. Time-course of fermentation of glucose-salts medium (original gravity 1.060) by *Saccharomyces cerevisiae* showing the effect of fresh trub . The points were the average of three independent fermentations and the variation never exceeded the 10% confidence limit. Key (○) no trub control; (●) 1 g fresh trub (□) 2 g fresh trub.

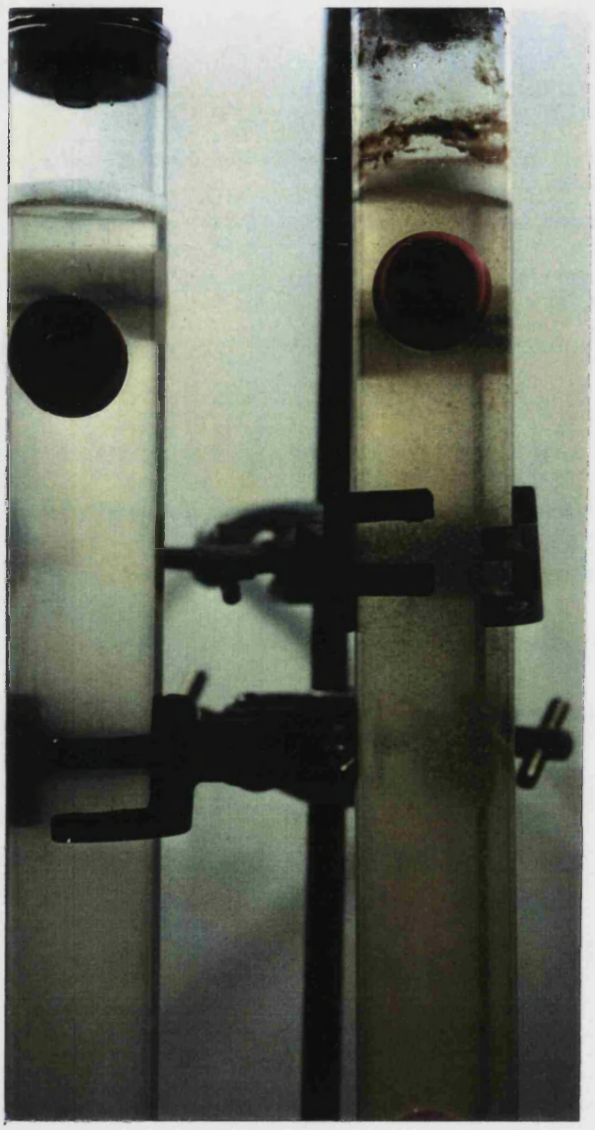


FIGURE 10. A photograph taken after 64 h of a fermentation of glucose-salts medium (original gravity 1.060°) by *Saccharomyces cerevisiae* showing greater yeast activity in the trub-containing fermentation on the right than in the control fermentation on the left.

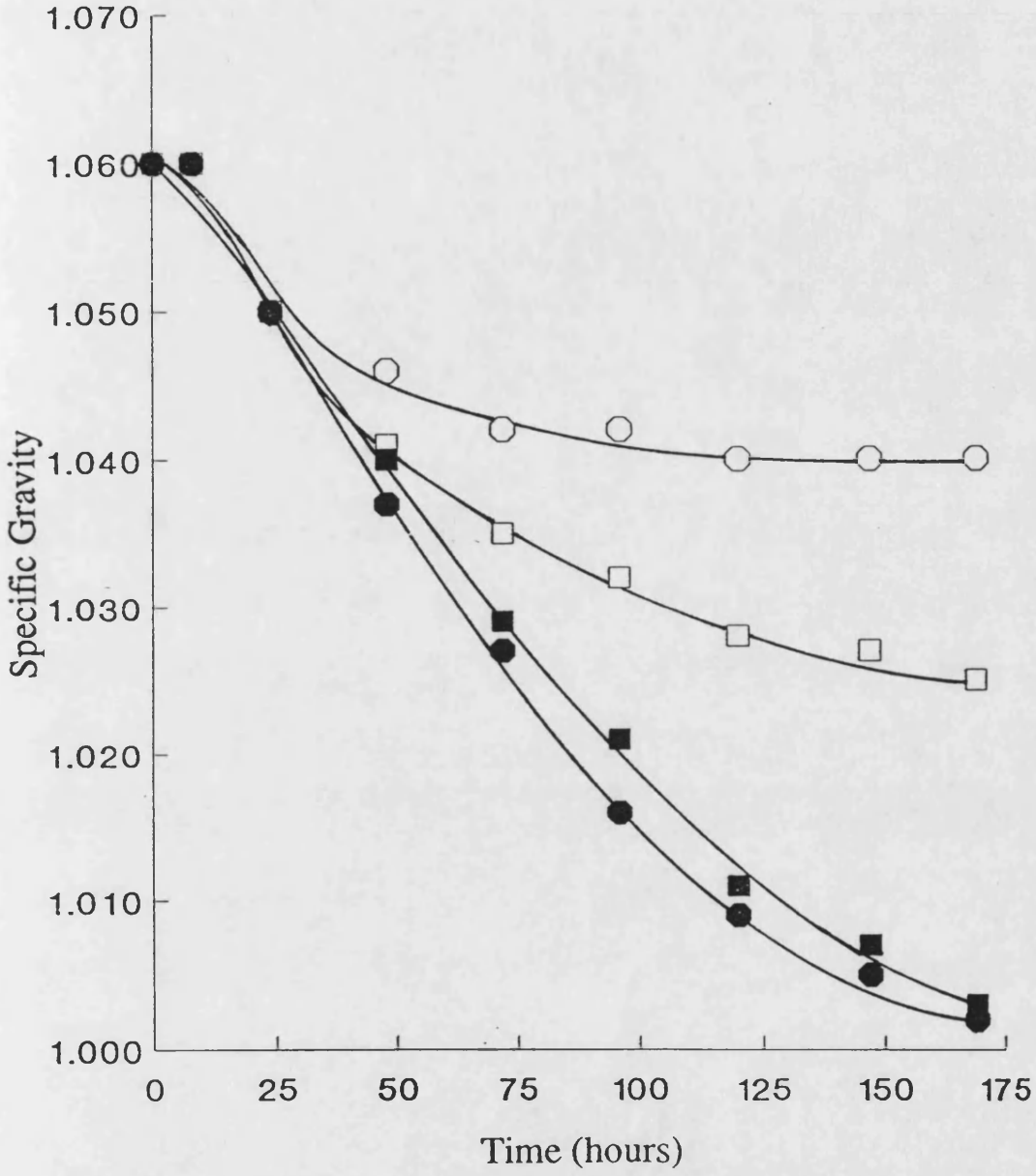


FIGURE 11. Time-course of fermentation of glucose-salts medium (original gravity 1.060) by *Saccharomyces cerevisiae* showing the effect of the water-insoluble and water-soluble fractions of trub. The values plotted were the average of three independent fermentations and the variation never exceeded the 10% confidence limit. Key: (○) no trub control; (●) 1 g trub; (□) water-soluble extract; (■) 1 g water-insoluble extract.

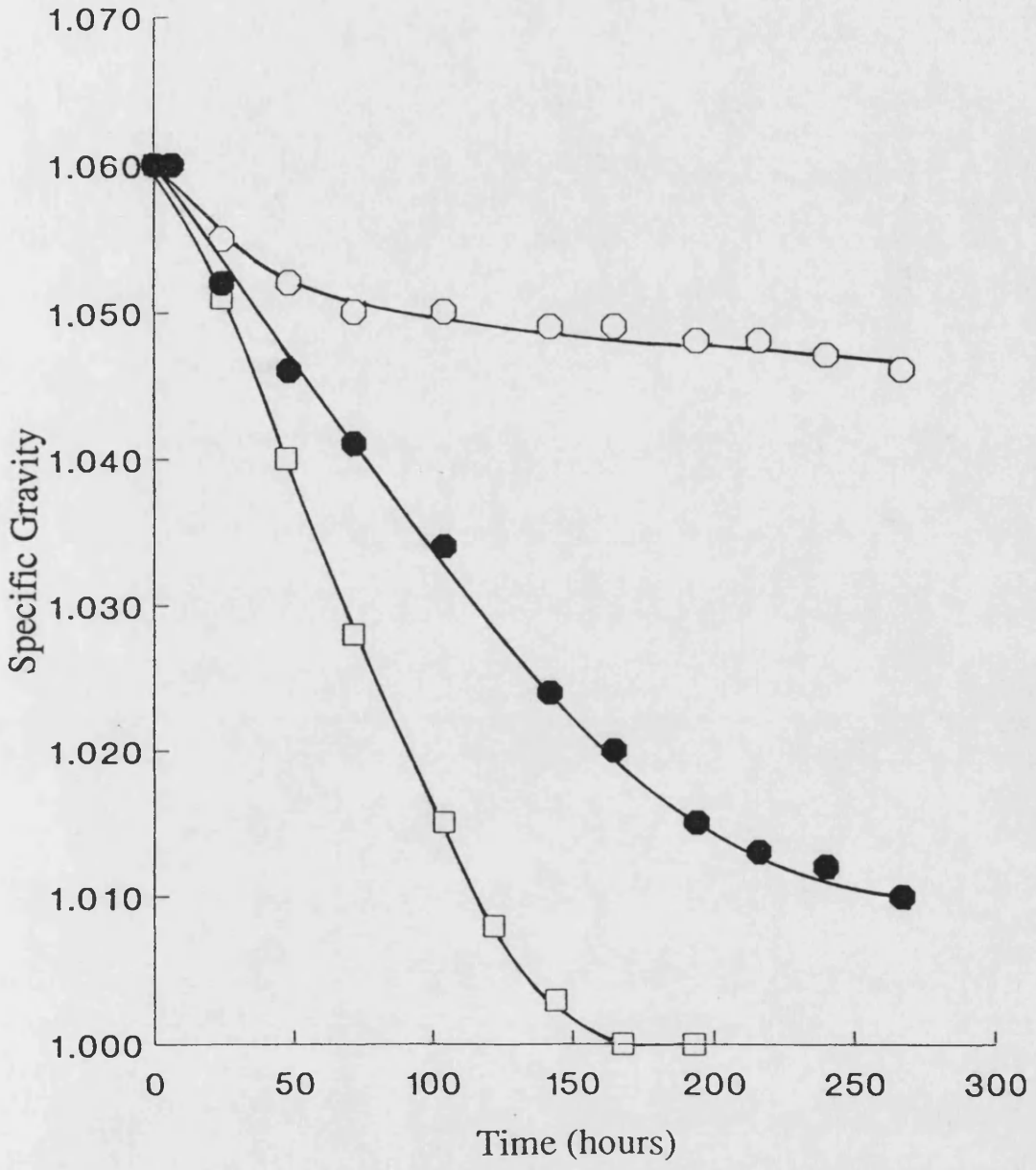


FIGURE 12. Time-course of fermentation of glucose-salts medium (original gravity 1.060) by *Saccharomyces cerevisiae* showing the effect of lipid-extracted trub. The values plotted were the average of three independent fermentations and the variation never exceeded the 10% confidence limit. Key: (○) no trub control; (●) 1 g lipid-extracted trub; (□) 1 g trub.

eventually stuck at a specific gravity of 1.035 (Figure 13). The ashed fraction from trub was initially inhibitory to the fermentation of glucose-salts medium (original gravity 1.060). Ashed trub allowed marginally more of the glucose to be fermented than the control fermentation but fermentations had stuck at a specific gravity of 1.040 (Figure 14). EDTA-treated trub stimulated fermentation of the medium. However the stimulative effect was much less than that of whole trub and fermentations to which EDTA-treated trub had been added stuck at a specific gravity of 1.030 (Figure 15). EDTA-treated lipid-extracted trub stimulated fermentation to a greater degree than control fermentations although the fermentations stuck at a specific gravity of 1.040 (Figure 16). EDTA-treated lipid-extracted trub was less stimulative to fermentation of glucose-salts medium (original gravity 1.060) than either EDTA-treated or lipid-extracted trub.

In summary all trub fractions stimulated the fermentation of glucose-salts medium (original gravity 1.060) by *Sacch. cerevisiae* to a greater degree than the control fermentations. However all trub fractions were less stimulative than whole trub. An order for the ability of trub fractions to stimulate fermentations, most beneficial first, would run: (1) water insoluble trub; (2) lipid-extracted trub; (3=) water soluble trub extract and EDTA-treated trub; (5) lipid extract of trub; (6=) EDTA-treated lipid-extracted trub and ashed trub.

Effect of supplements

The addition of pure lipid, or, neutralised activated charcoal, did not have a stimulative effect on the fermentation of glucose-salts medium (original gravity 1.060) by *Saccharomyces cerevisiae* (Figures 17 and 18). Additon of mineral salts, 0.1 p.p.m.copper, 0.1 p.p.m. manganese and 0.64 p.p.m. zinc (the amount found in trub) did not stimulate the fermentation of glucose-salts medium (original gravity 1.060) by *Saccharomyces cerevisiae* (Figures 19 (a), (b) and (c)).

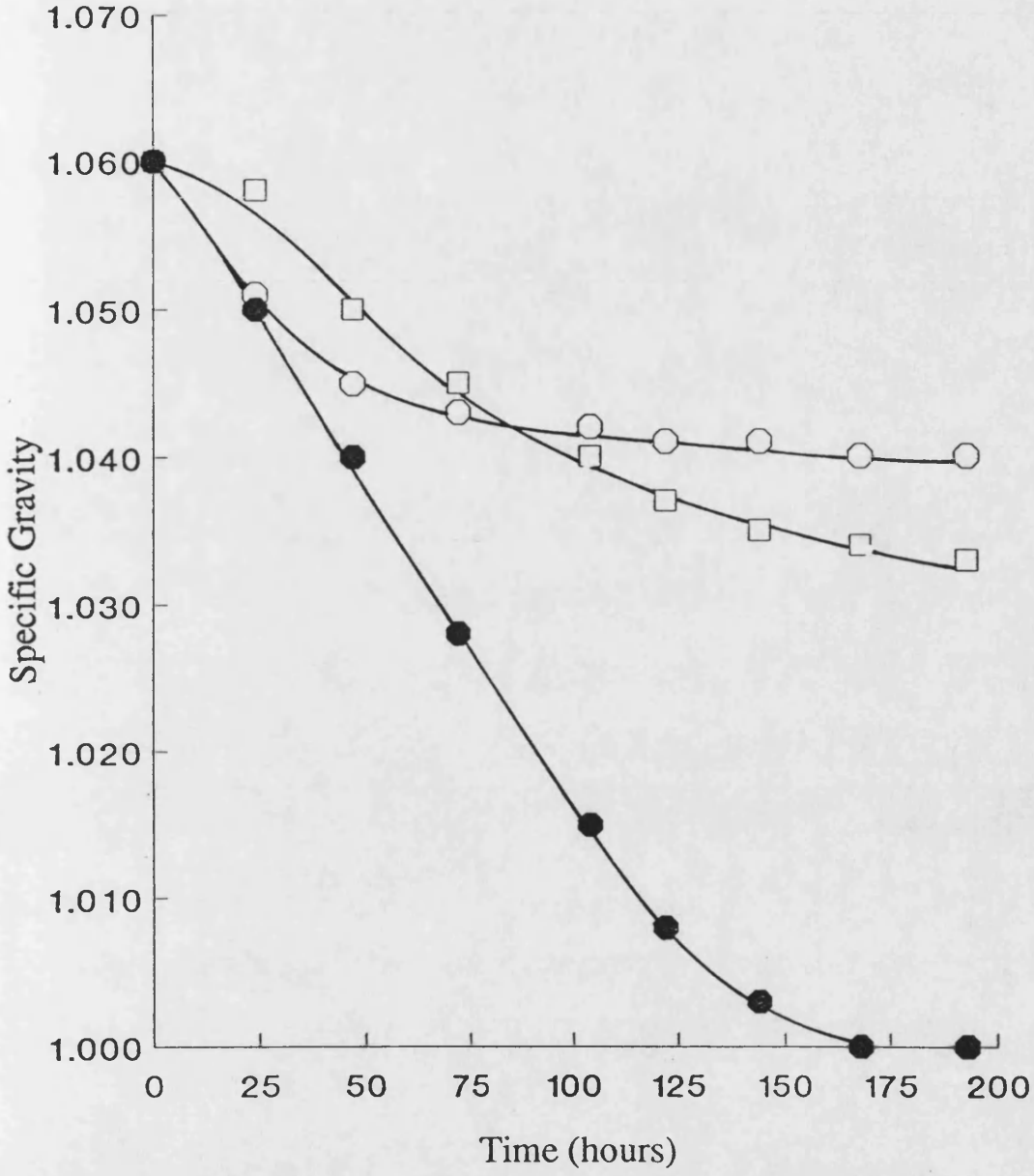


FIGURE 13. Time-course of fermentation of glucose-salts medium (original gravity 1.060) by *Saccharomyces cerevisiae* showing the effect of the lipid extract from 1 g trub. The values plotted were the average of three independent fermentations and the variation never exceeded the 10% confidence limit. Key: (○) no trub control; (□) lipid extract from 1 g trub; (●) 1 g trub.

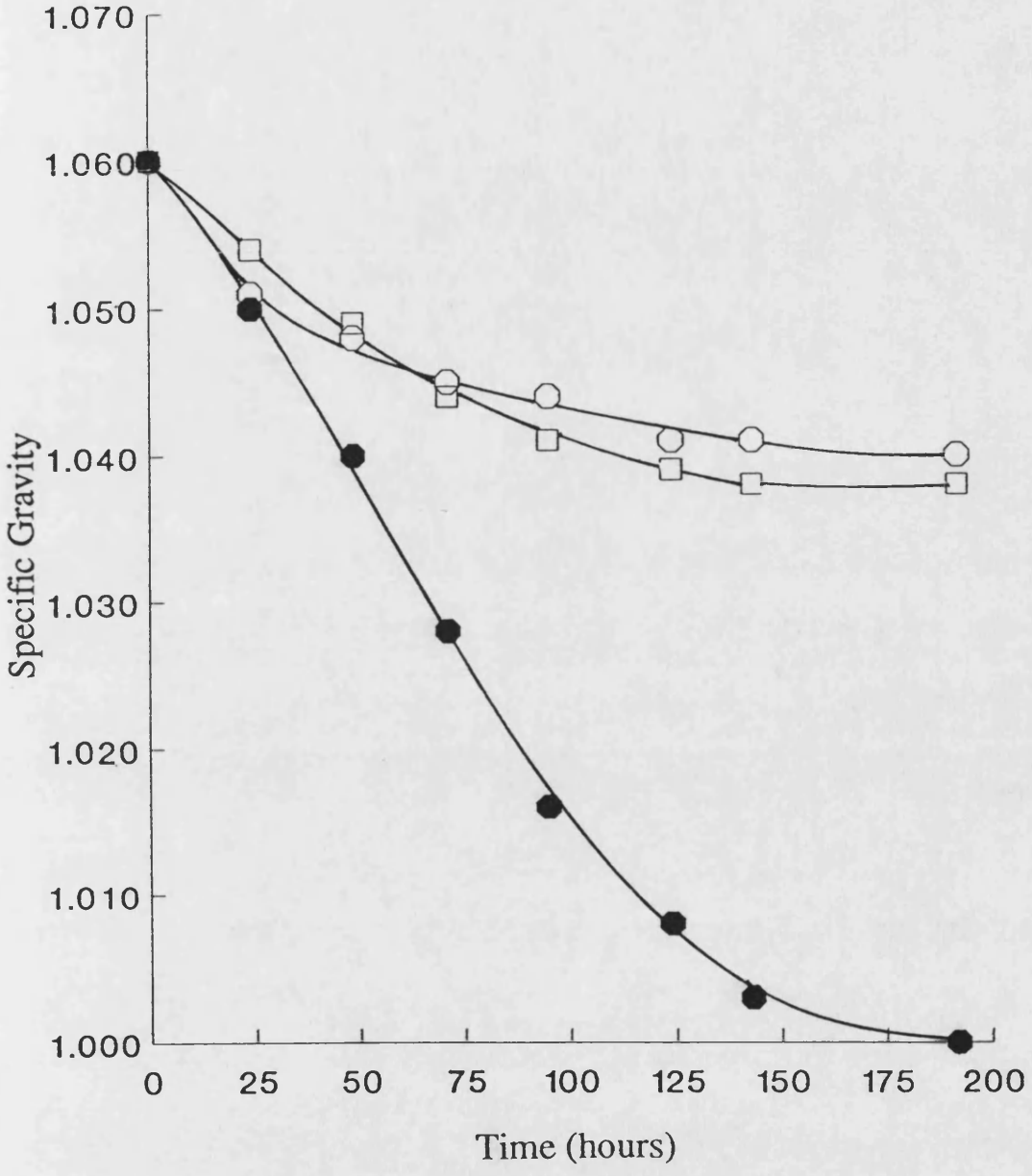


FIGURE 14. Time-course of fermentation of glucose-salts medium (original gravity 1.060) by *Saccharomyces cerevisiae* showing the effect of ashed trub. The values plotted were the average of three independent fermentations and the variation never exceeded the 10% confidence limit. Key: (○) no trub control; (□) ashes from 1 g trub; (●) 1 g trub.

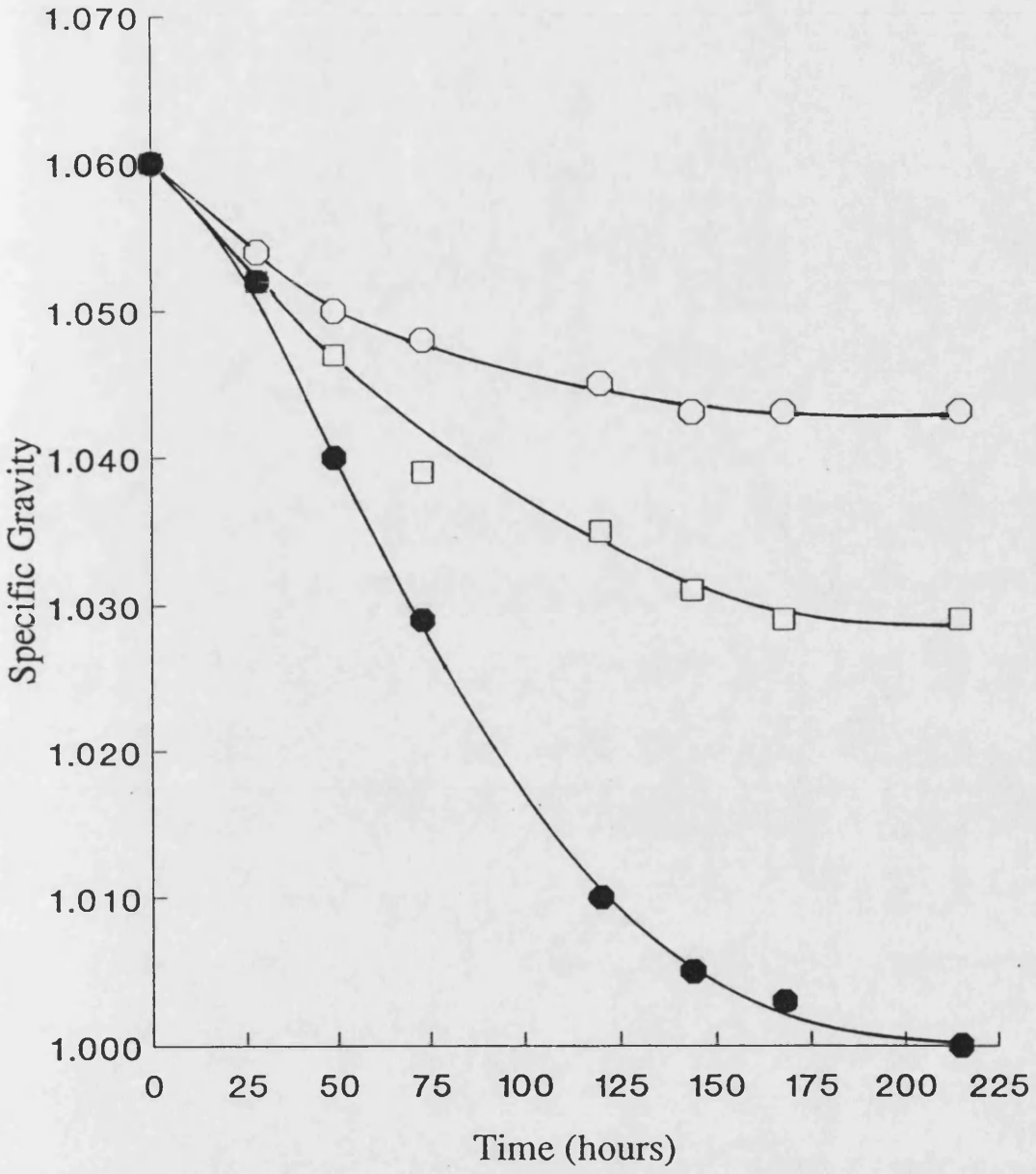


FIGURE 15. Time-course of fermentation of glucose-salts medium (original gravity 1.060) by *Saccharomyces cerevisiae* showing the effect of 1 g EDTA-treated trub. The values plotted were the average of three independent fermentations and the variation never exceeded the 10% confidence limit. Key: (○) no trub control; (□) 1 g EDTA-treated trub; (●) 1 g trub.

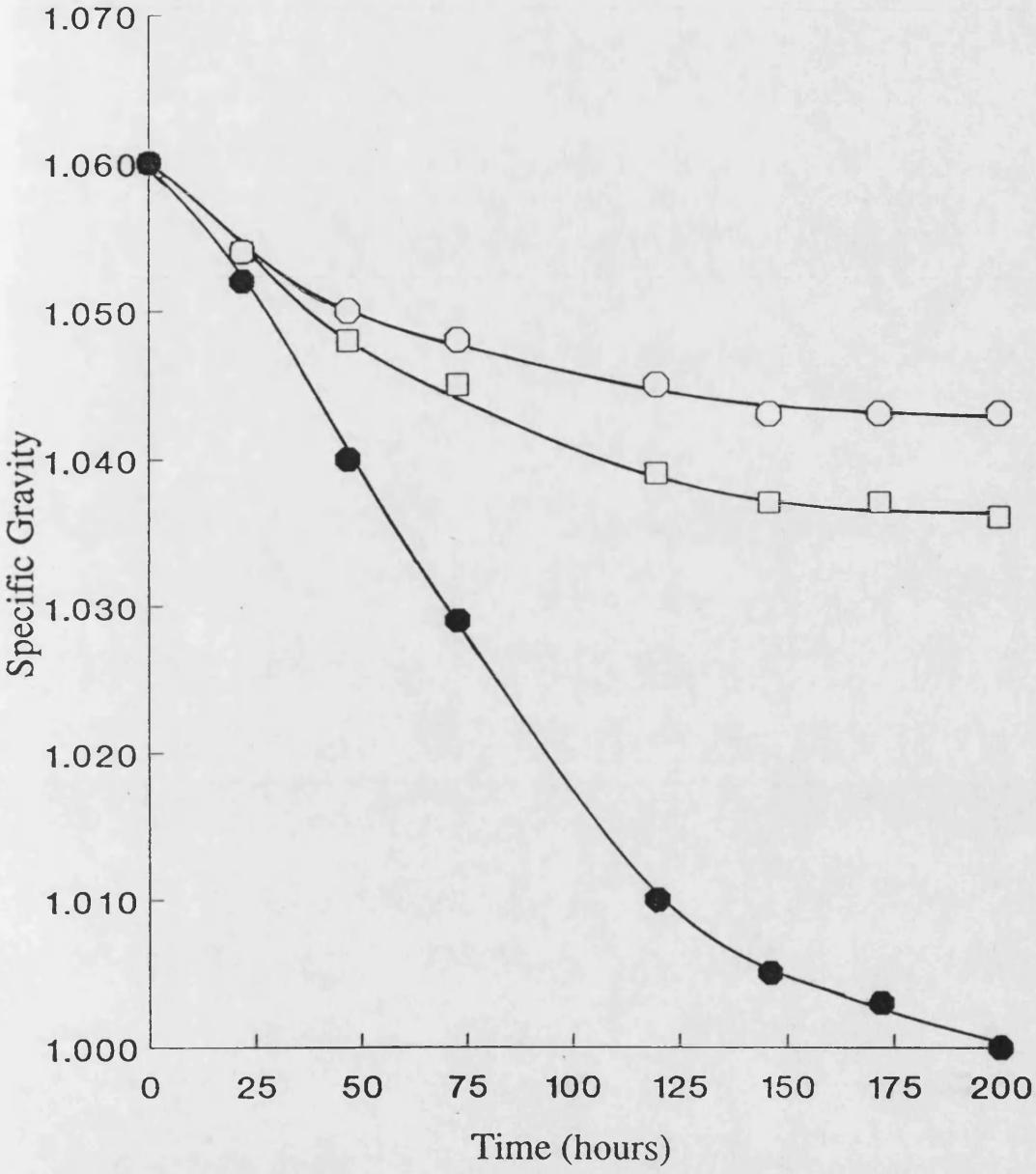


FIGURE 16. Time-course of fermentation of glucose-salts medium (original gravity 1.060) by *Saccharomyces cerevisiae* showing the effect of 1 g EDTA-treated lipid-extracted trub. The values plotted were the average of three independent fermentations and the variation never exceeded the 10% confidence limit. Key: (○) no trub control; (□) 1 g EDTA-treated lipid-exracted trub; (●) 1 g trub.

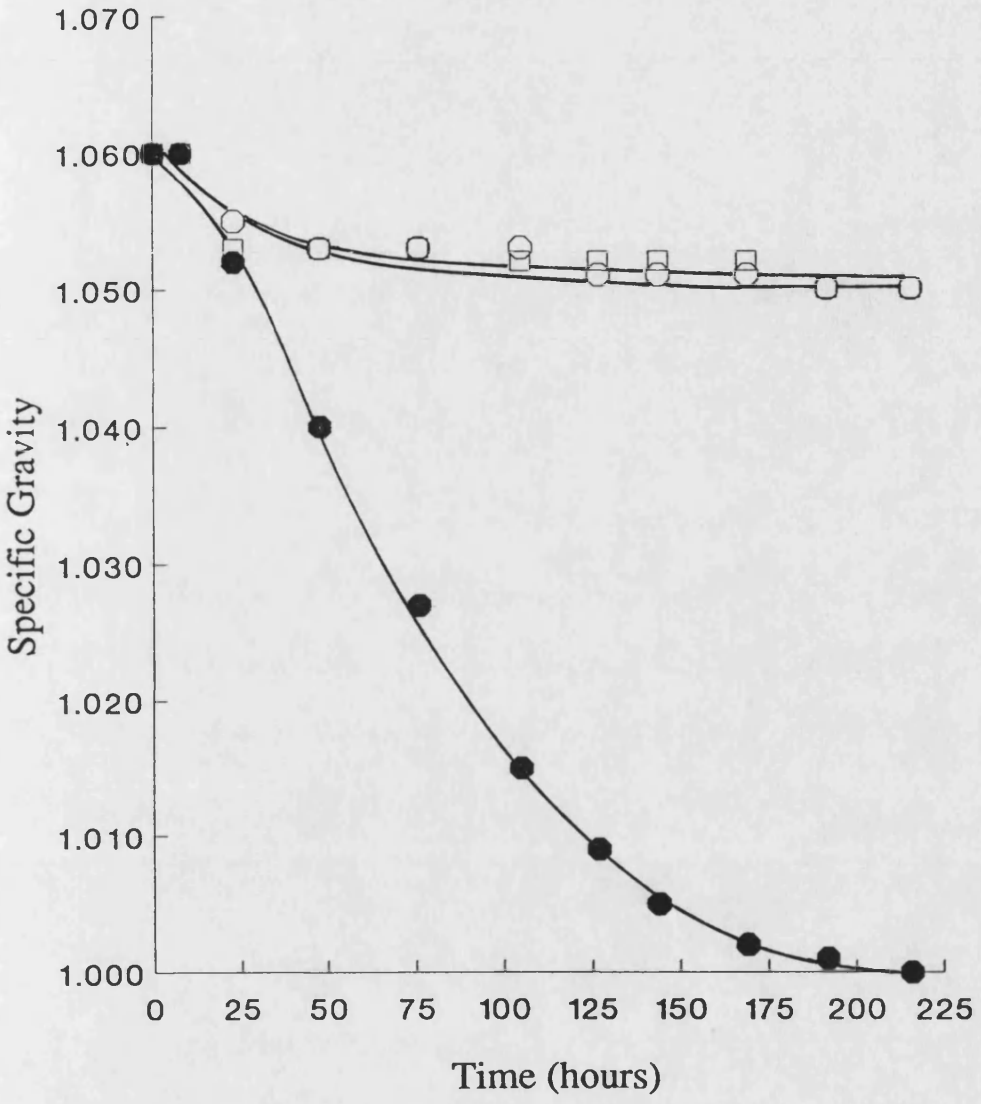


FIGURE 17. Time-course of fermentation of glucose-salts medium (original gravity 1.060) by *Saccharomyces cerevisiae* showing the effect of pure unsaturated fatty acid and sterol lipid supplements. The unsaturated fatty acid supplement ~~used~~ was linoleic acid (5.4 mg) and the sterol supplement was β-sitosterol (1 mg), which were dissolved in ethanol and added to the medium. The amounts of linoleic acid and β–sitosterol added corresponded to the average amount of each lipid found in trub. The values plotted were the average of three independent fermentations and the variation never exceeded the 10% confidence limit. Key: (○) no trub control; (□) 5.4 mg linoleic acid and 1 mg β–sitosterol; (●) 1 g trub.

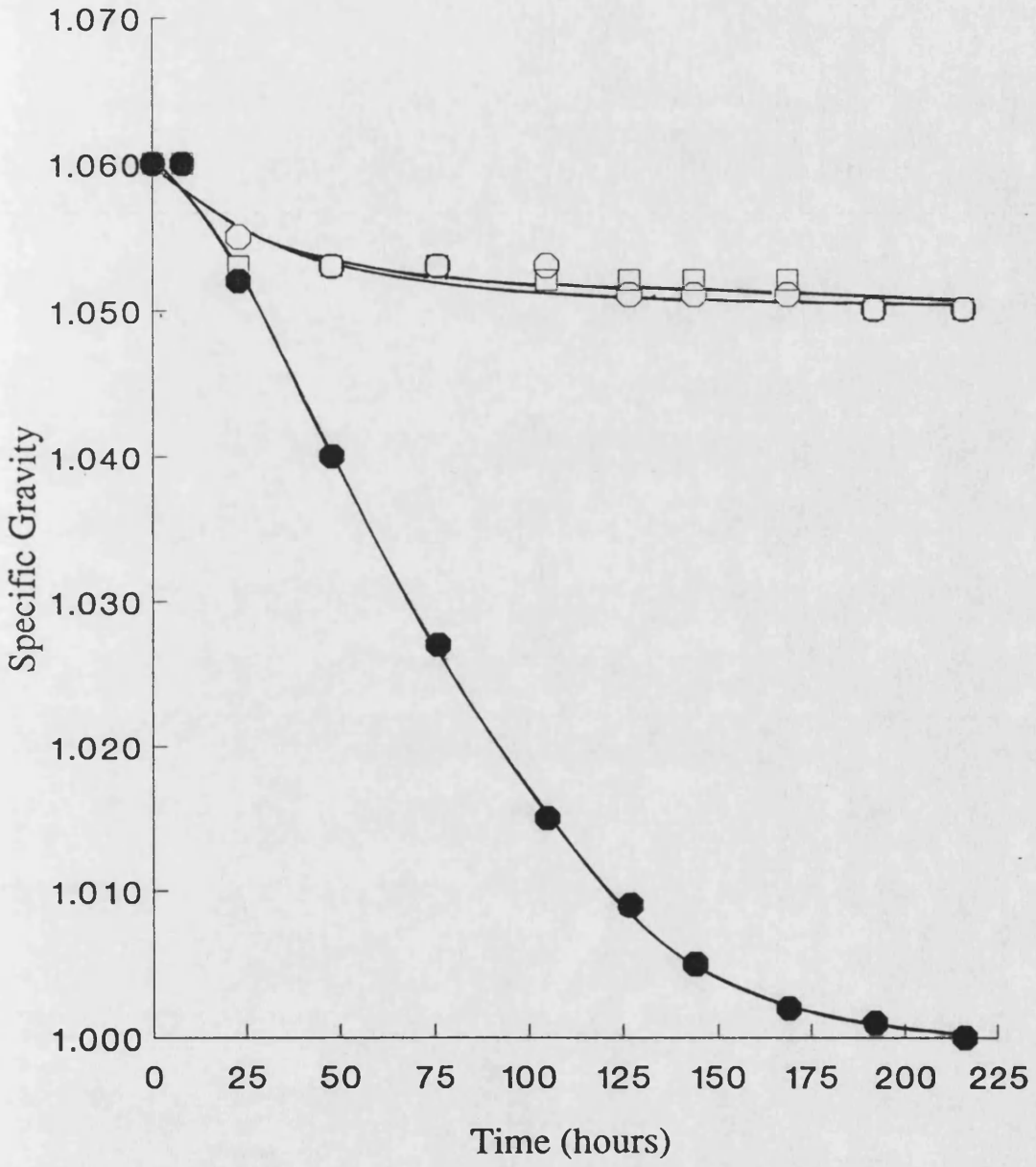
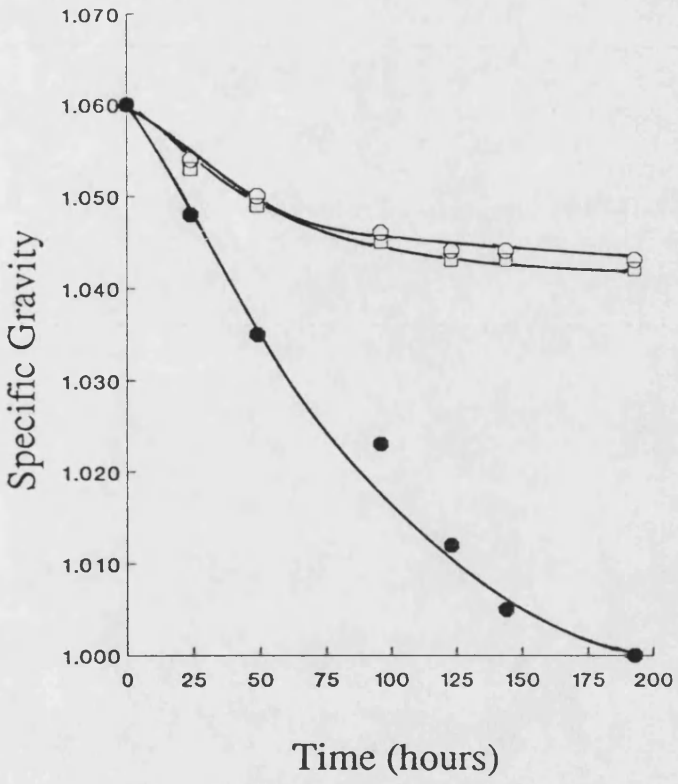
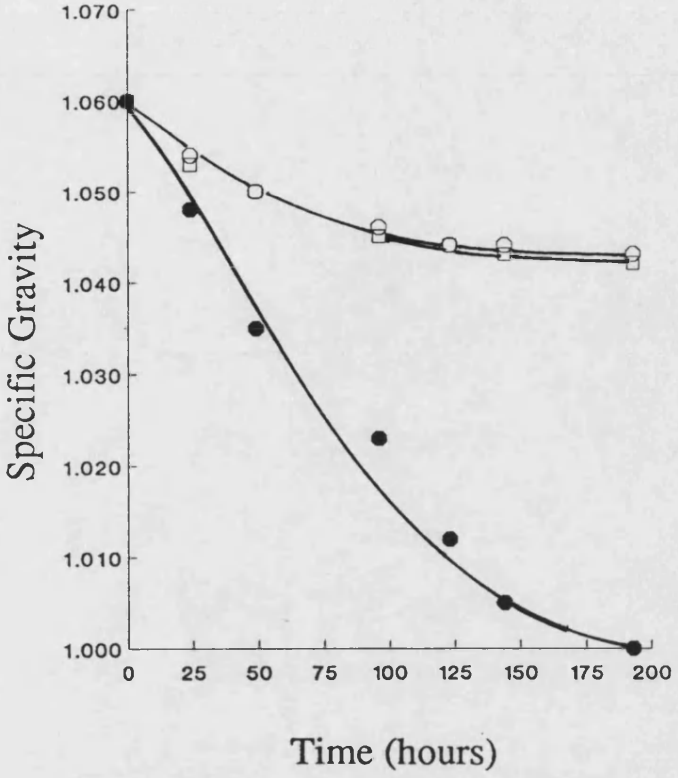


FIGURE 18. Time-course of fermentation of glucose-salts medium (original gravity 1.060) by *Saccharomyces cerevisiae* showing the effect of neutralised activated charcoal. The values plotted were the average of three independent fermentations and the variation never exceeded the 10% confidence limit. Key: (○) no trub control; (□) 1 g neutralised activated charcoal; (●) 1 g trub.

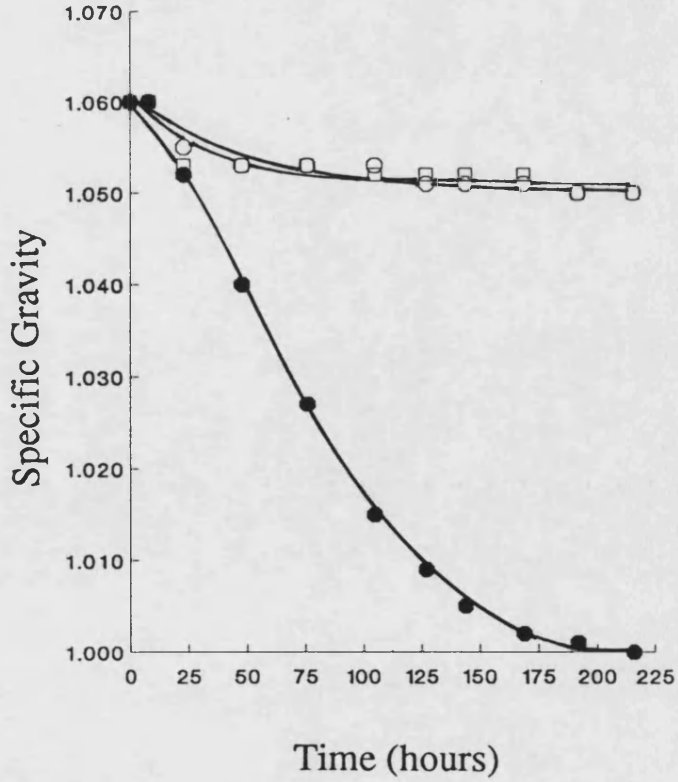


(a)

FIGURE 19. Time-course of fermentation of glucose-salts medium (original gravity 1.060°) by *Saccharomyces cerevisiae* showing the effect of copper (Figure 19 (a)), manganese (Figure 19 (b)) and zinc ions (Figure 19 (c)). Copper was added as a solution of Cu(II)SO_4, manganese as a solution of $MnCl_2$ and zinc as a solution of $ZnSO_4$. All salt solutions were added to media before autoclaving. The values plotted were the average of three independent fermentations and the variation never exceeeded the 10% confidence limit. Key: (○) no trub control; (●) 1 g trub; (□) (a) 0.1 p.p.m. copper; (b) 0.1 p.p.m. manganese; (c) 0.64 p.p.m. zinc.


1.070
1.060
1.050
1.040
1.030
1.020
1.010
1.000
Specific Gravity
0
25
50
75
100
125
150
175
200
Time (hours)

(b)


1.070
1.060
1.050
1.040
1.030
1.020
1.010
1.000
Specific Gravity
0
25
50
75
100
125
150
175
200
225
Time (hours)

(c)

Effect of repitching yeasts from fermentations into fresh medium

Yeasts harvested from either control fermentations or trub-containing fermentations of glucose-salts medium (original gravity 1.060) were washed twice with distilled water as described in the Methods section and held at 4°C for six hours prior to repitching into fresh glucose-salts medium (original gravity 1.060). Yeast from trub-containing fermentations fermented glucose-salts medium (original gravity 1.060) in the presence of trub to a specific gravity of 1.004 . In the absence of trub yeast from trub-containing fermentations exhibited a slower fermentation rate (Figure 20). Yeast from control fermentations fermented glucose-salts medium (original gravity 1.060) in the absence of trub at a much slower rate than yeasts from trub-containing fermentations (Figures 20 and 21). Repitched yeast from control fermentations fermented glucose-salts medium (original gravity 1.060) at a much slower rate than yeast from trub-containing fermentations yet the medium was attenuated to the same degree (Figures 20 and 21). Fermentations of repitched yeasts from control fermentations were characterised by their long lag-phase regardless of whether trub was present or not (Figure 21).

FERMENTATION OF BREWER'S WORT

Effect of trub

The addition of 1 g trub to brewer's wort (original gravity 1.060) had a stimulative effect on fermentation by *Saccharomyces cerevisiae* (Figure 22). However fermentations to which 100 mg and 250 mg of trub were added did not ferment any quicker than control fermentations. Fermentations to which 1 g trub had been added fermented to a specific gravity of 1.010 after 96 h whereas control fermentations and those fermentations to which 100 mg or 250 mg trub had been added had not reached 1.010° specific gravity after 145 h (Figure 22). As in the case of fermentations with glucose-salts media when the specific gravity of the wort was raised from 1.060 to 1.080 and 1.100 , respectively, the stimulative effect of trub on fermentation was

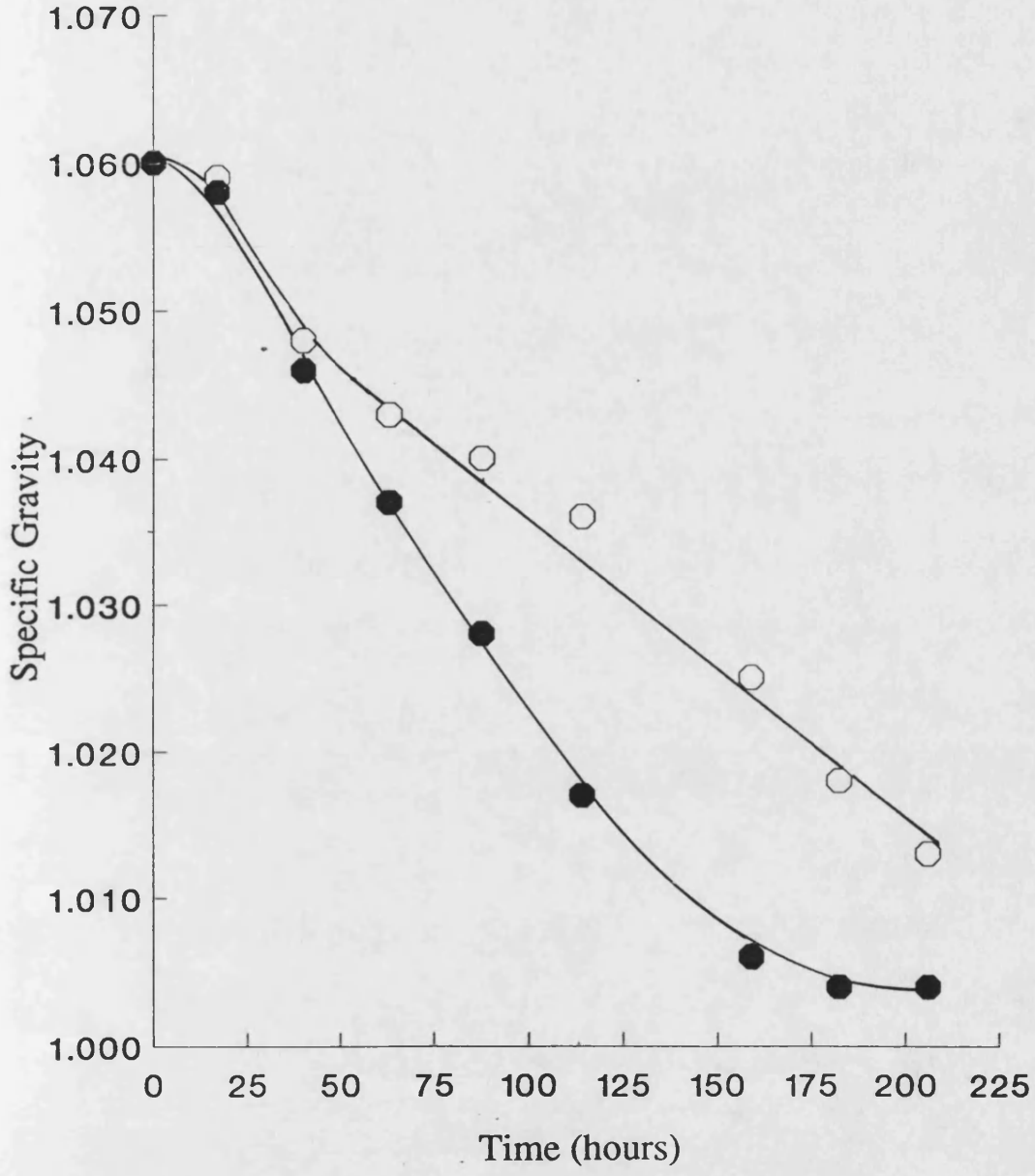


FIGURE 20. Time-course of fermentation of glucose-salts medium (original gravity 1.060) fermented by *Saccharomyces cerevisiae* harvested from trub-containing fermentations showing the effect of trub. The values plotted were the average of three independent fermentations and the variation never exceeeded the 10% confidence limit. Key: (◯) no trub control; (●) 1 g trub.

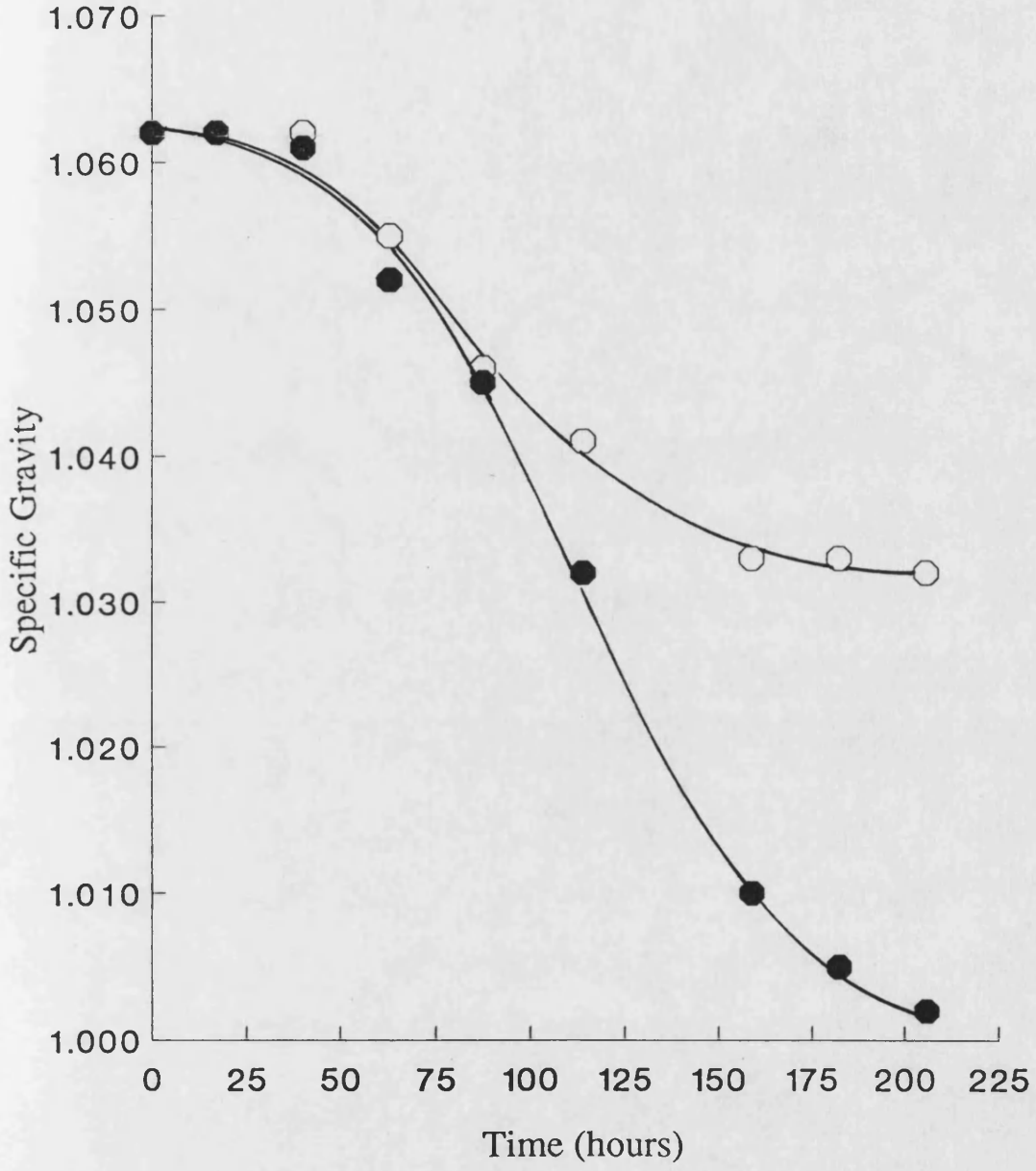


FIGURE 21. Time-course of fermentation of glucose-salts medium (original gravity 1.060) by *Saccharomyces cerevisiae* harvested from control fermentations of glucose-salts medium (original gravity 1.060) showing the effect of trub. The values plotted were the average of three independent fermentations and the variation never exceeded the 10% confidence limit. Key: (○) no trub control; (●) 1 g trub.

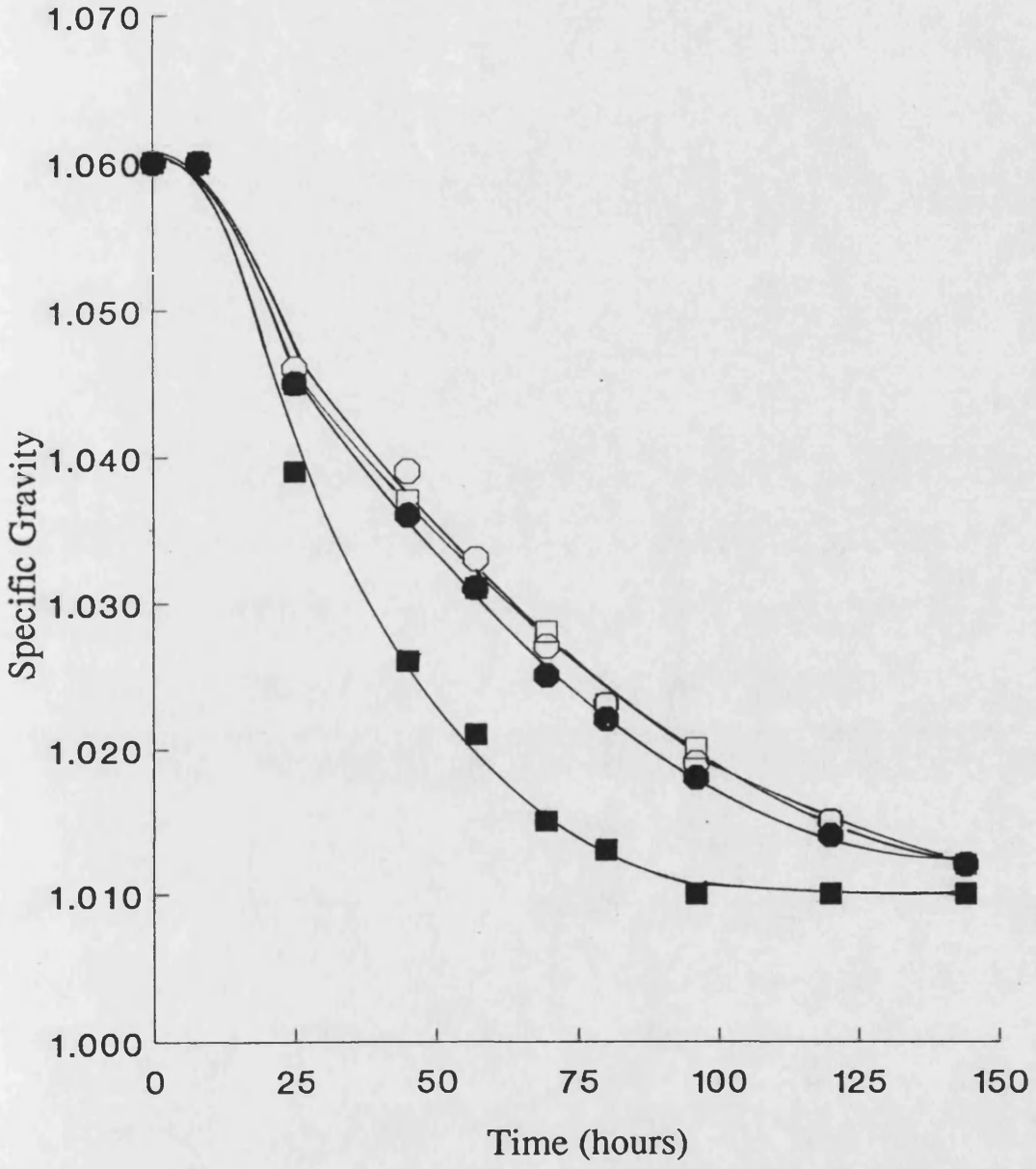


FIGURE 22. Time-course of fermentation of brewer's wort (original gravity 1.060) by *Saccharomyces cerevisiae* showing the effect of trub. The values plotted were the average of three independent fermentations and the variation never exceeded the 10% confidence limit. Key: (○) no trub control; (●) 100 mg trub; (□) 250 mg trub; (■) 1 g trub.

more pronounced (Figures 23 and 24). This effect exhibited itself as faster and greater attenuation of the wort when trub was present. The specific gravities for fermentations of worts of specific gravities 1.080 and 1.100 were 1.014 and 1.030 , respectively. Turbid wort (original gravity 1.080), collected, dispensed and sterilised as described in the Methods except that the clarification step was omitted, was attenuated by *Sacch. cerevisiae* marginally faster than the control fermentations. However fermentations to which trub had been added attenuated more rapidly than turbid wort fermentations (Figure 25).

Effect of trub fractions

Trub fractions showed a range of abilities to stimulate the fermentation of brewer's wort by *Sacch. cerevisiae*. Lipid-extracted trub was marginally less stimulative than trub when brewer's wort (original gravity 1.060) was fermented by *Sacch. cerevisiae* (Figure 26). Fermentations to which lipid-extracted trub had been added fermented to a specific gravity of 1.010 after 168 h compared to trub-containing fermentations which fermented to a specific gravity of 1.008 after 143 h. Control fermentations fermented to a specific gravity of 1.012 after 192 h. Lipid-extracted EDTA-treated trub was more stimulative to fermentation of brewer's wort (original gravity 1.080) than either the ashed fraction of trub or the soluble fraction of ashed trub (Figures 27, 28 and 29) Fermentations to which lipid-extracted EDTA-treated trub had been added fermented to a specific gravity of 1.016 after 143 h compared to a specific gravity of 1.018 after 212 h for the ashed fraction of trub and 1.021 after 212 h for the soluble fraction of ashed trub, respectively. All trub fractions were more stimulative to the fermentation than the control but none stimulated fermentation to the same degree as whole trub.

Effect of supplements

Activated charcoal stimulated the fermentation of brewer's wort (original gravity 1.080) by *Sacch. cerevisiae* but the stimulative effect was only noticeable

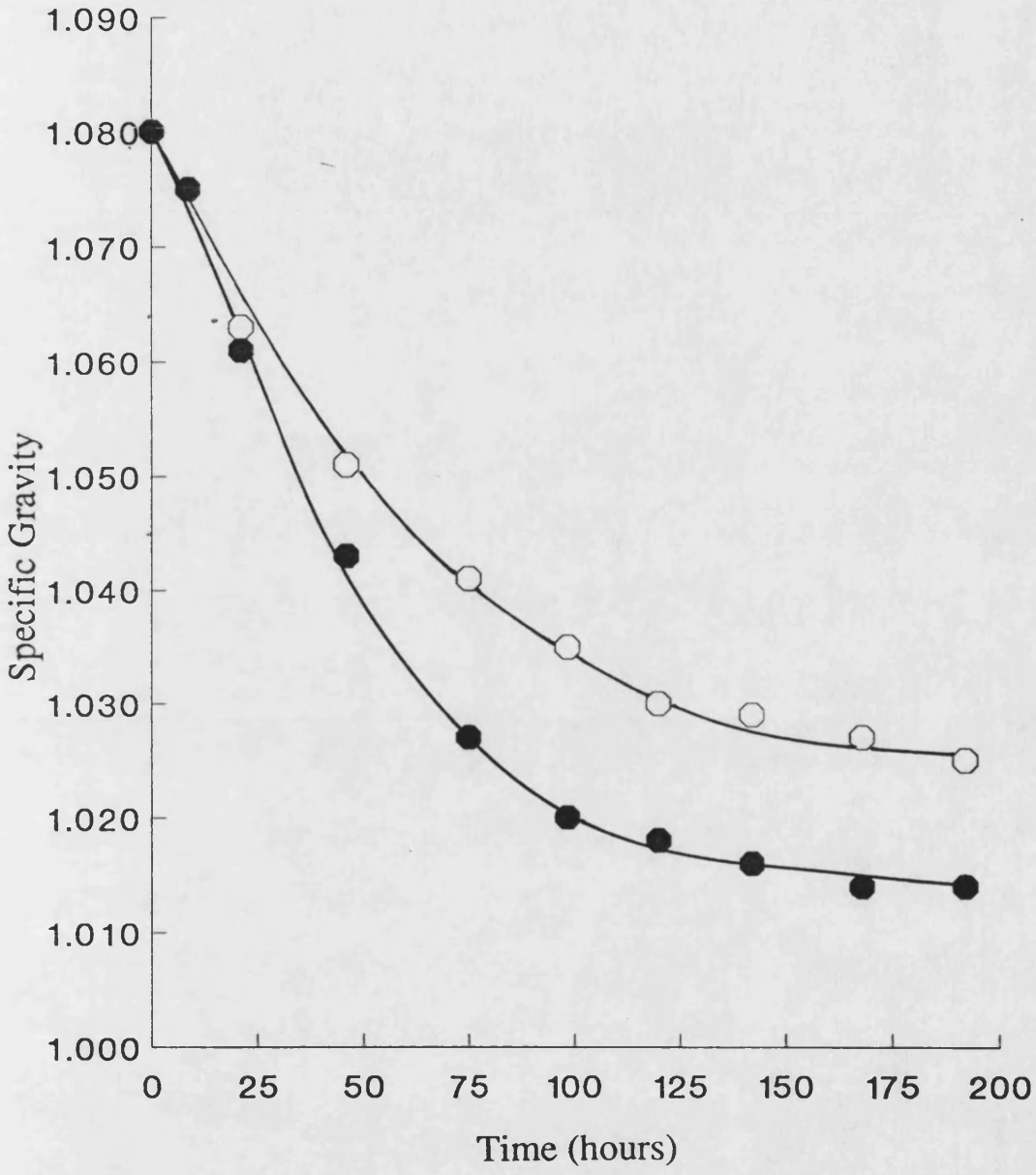


FIGURE 23. Time-course of fermentation of brewer's wort (original gravity 1.080) by *Saccharomyces cerevisiae* showing the effect of trub. The values plotted were the average of three independent fermentations and variation never exceeded the 10% confidence limit. Key (○) no trub control; (●) 1 g trub.

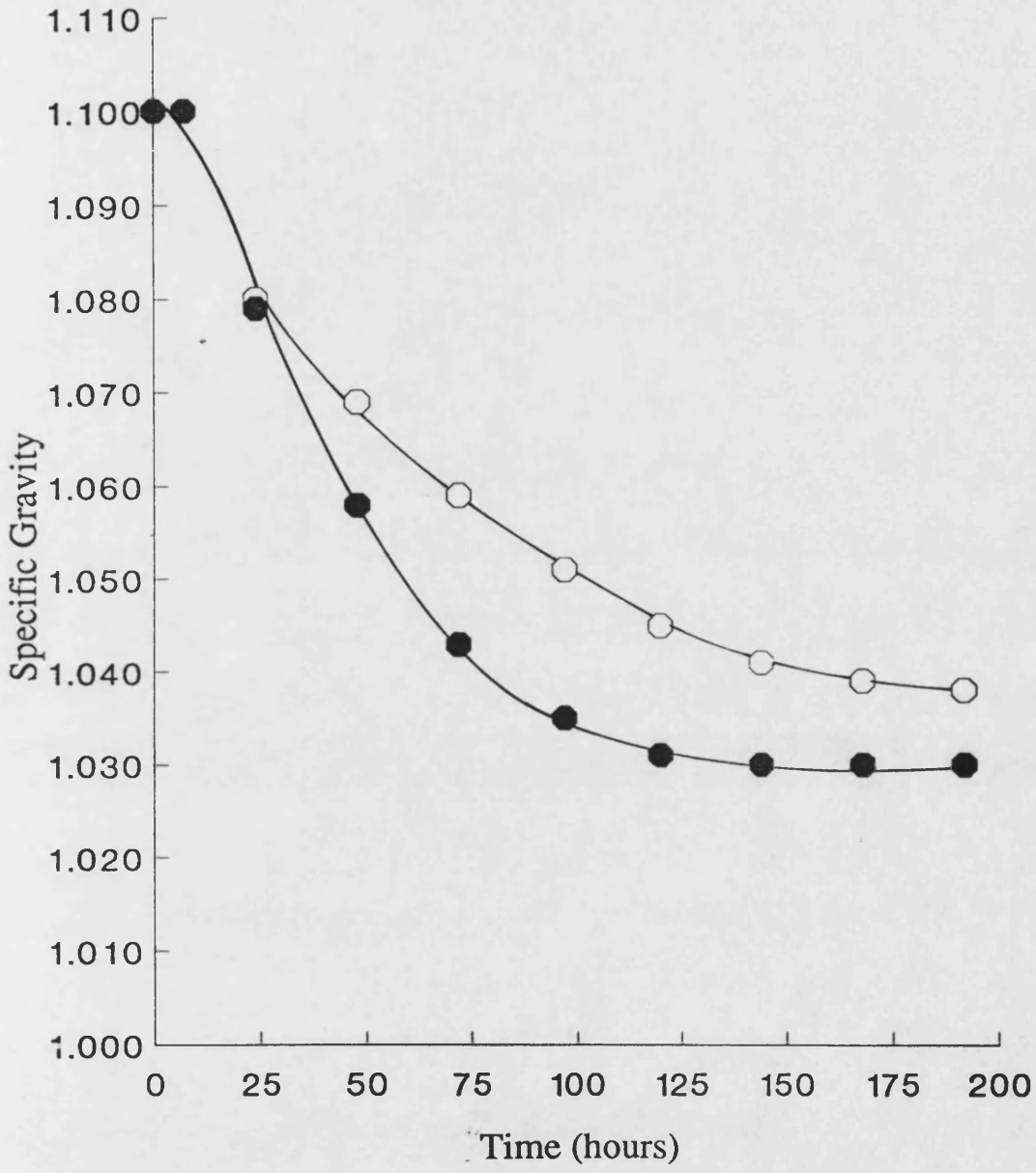


FIGURE 24. Time-course of fermentation of brewer's wort (original gravity 1.100) by *Saccharomyces cerevisiae* showing the effect of trub. The values plotted were the average of three independent fermentations and variation never exceeded the 10% confidence limit. Key (○) no trub control; (●) 1 g trub.

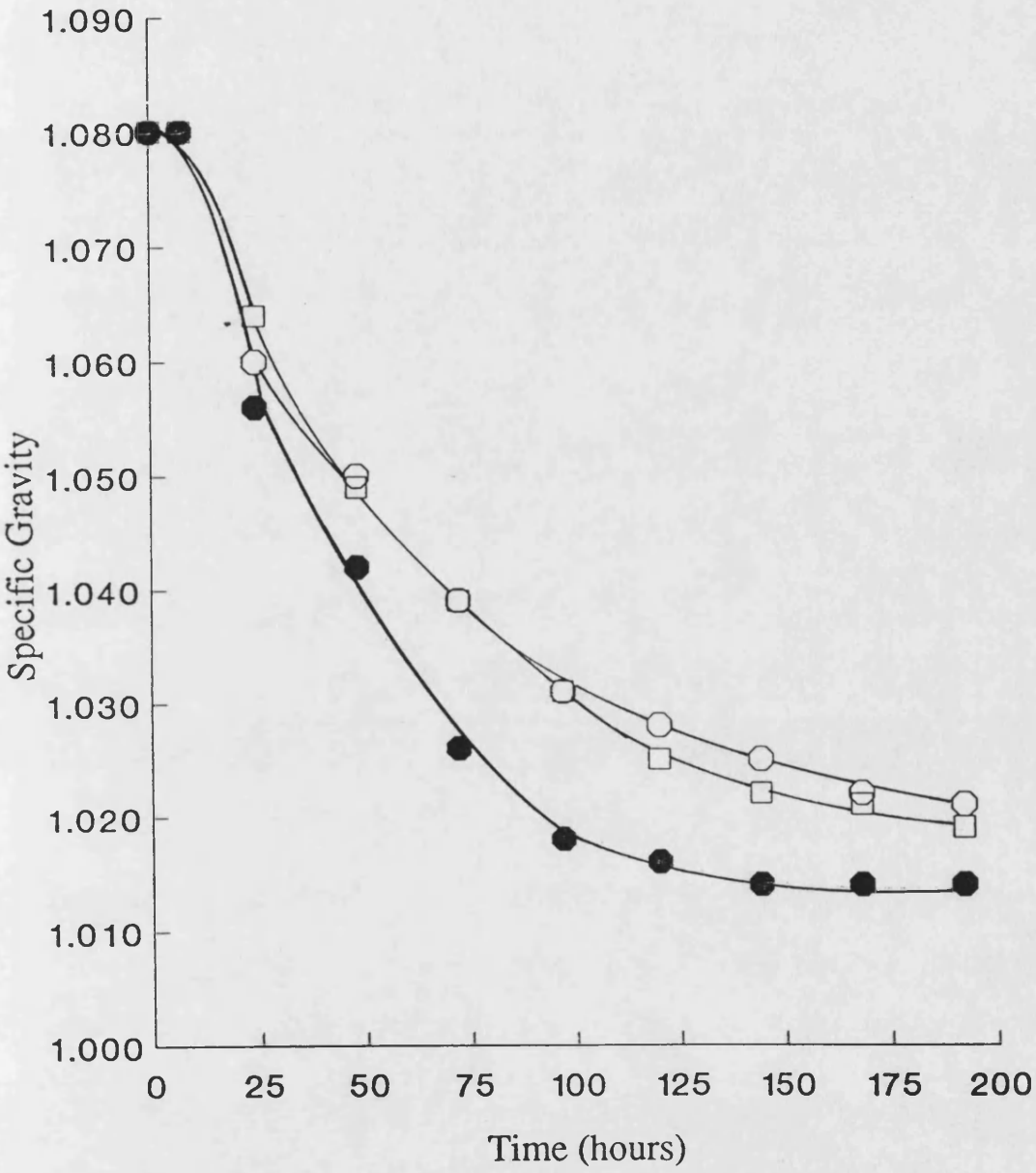


FIGURE 25. Time-course of fermentation of turbid brewer's wort (original gravity 1.080) by *Saccharomyces cerevisiae*. Turbid wort was collected as described in the Methods Section except that the clarification steps were omitted. The values plotted were the average of three independent fermentations and variation never exceeded the 10% confidence limit. Key: (◯) no trub control; (□) turbid wort; (●) 1 g trub.

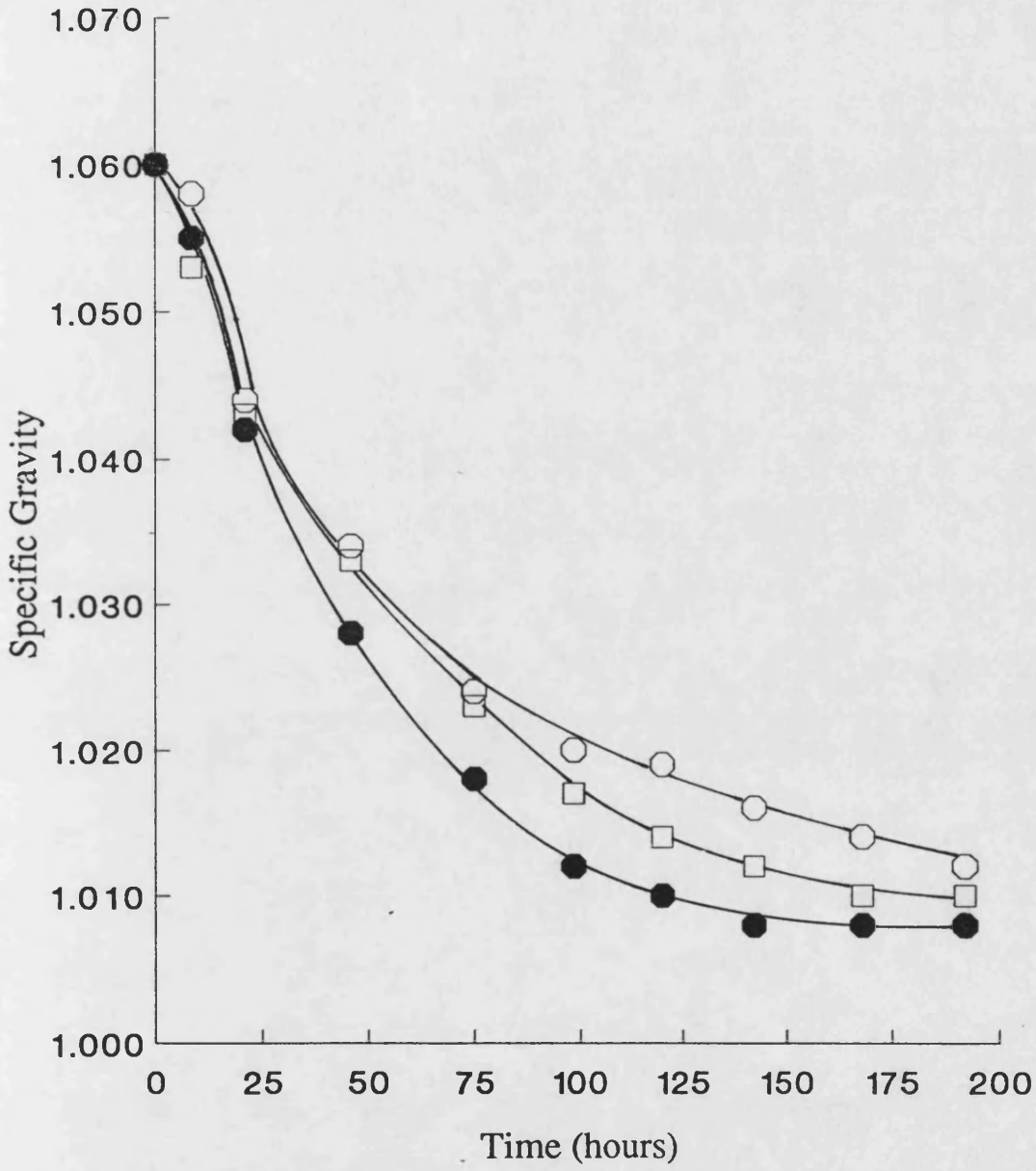


FIGURE 26. Time-course of fermentation of brewer's wort (original gravity 1.080) by *Saccharomyces cerevisiae* showing the effect of lipid-extracted trub. The values plotted were the average of three independent fermentations and the variation never exceeded the 10% confidence limit. Key: (○) no trub control; (□) 1 g lipid-extracted trub; (●) 1 g trub.

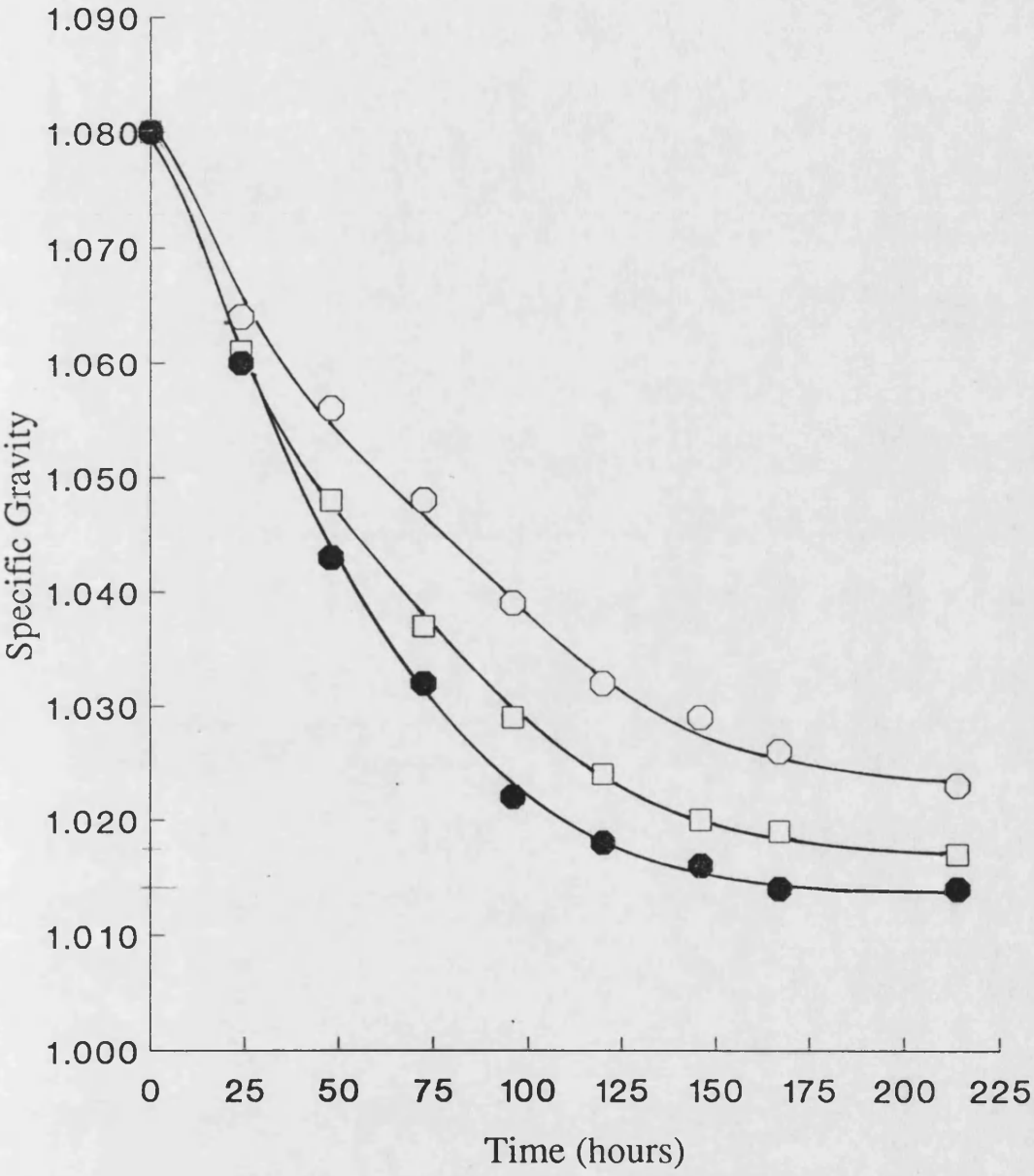


FIGURE 27. Time-course of fermentation of brewer's wort (original gravity 1.080) by *Saccharomyces cerevisiae* showing the effect of the ashed fraction of trub. The values plotted were the average of three independent fermentations and the variation never exceeded the 10% confidence limit. Key: (○) no trub control; (□) ashes from 1 g trub; (●) 1 g trub.

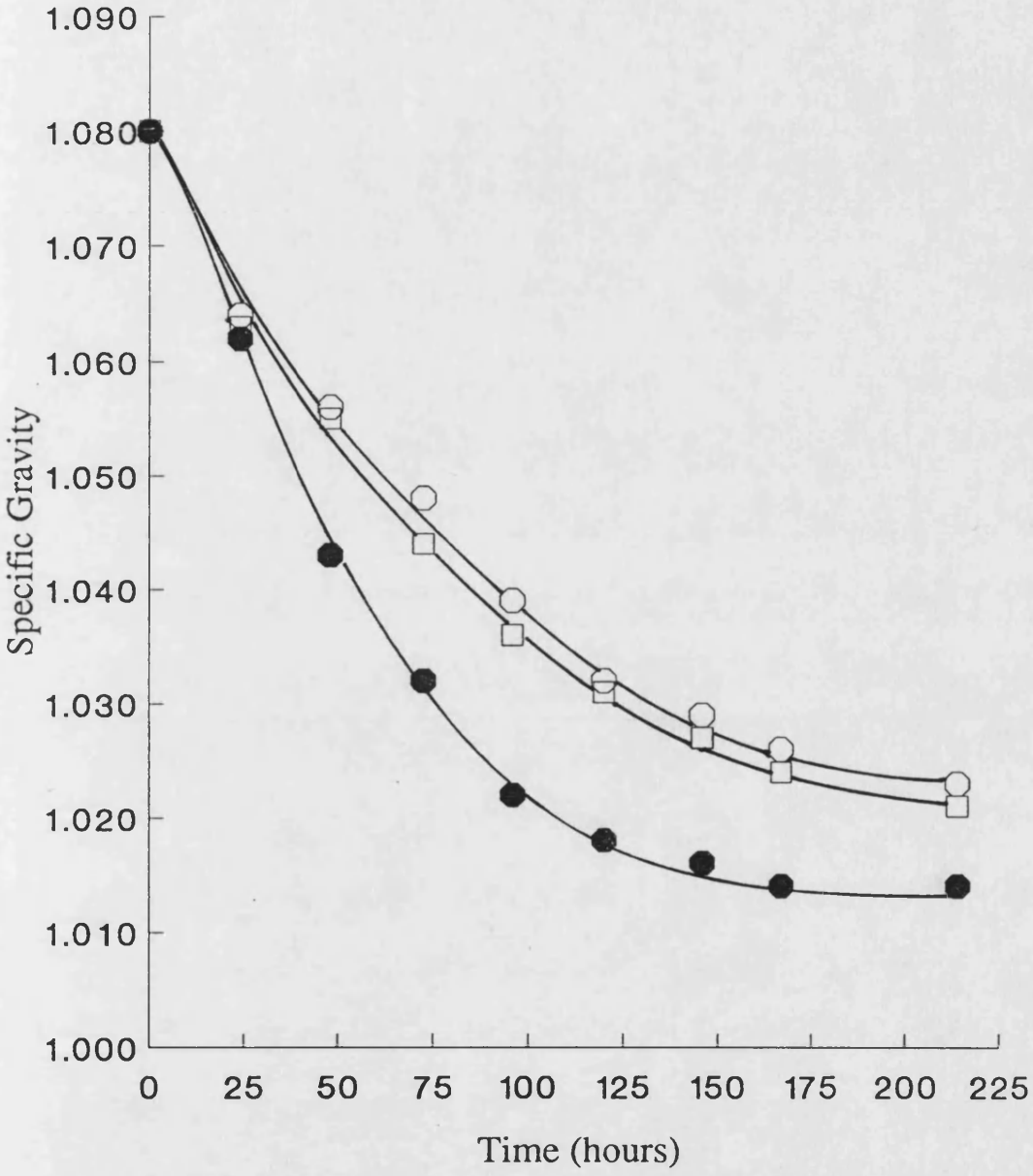


FIGURE 28. Time-course of fermentation of brewer's wort (original gravity 1.080) by *Saccharomyces cerevisiae* showing the effect of the soluble fraction of ashed trub. The values plotted were the average of three independent fermentations and the variation never exceeded the 10% confidence limit. Key: (○) no trub control; (□) soluble fraction from the ashes of 1 g trub; (●) 1 g trub.

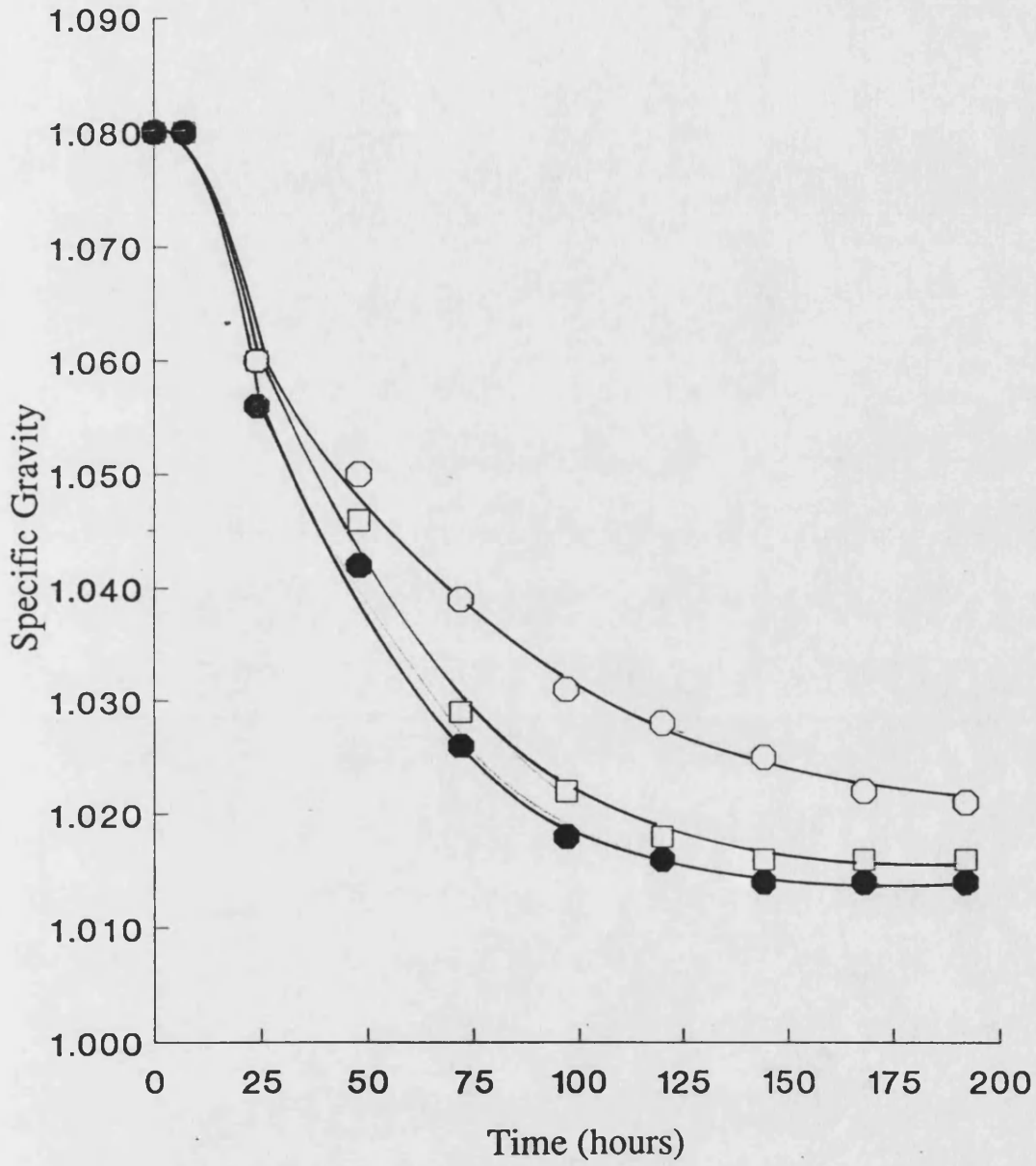


FIGURE 29. Time-course of fermentation of brewer's wort (original gravity 1.080) by *Saccharomyces cerevisiae* showing the effect of the lipid-extracted EDTA-treated trub. The values plotted were the average of three independent fermentations and the variation never exceeded the 10% confidence limit. Key: (○) no trub control; (□) 1 g lipid-extracted EDTA-treated trub; (●) 1 g trub.

the last third of the fermentation (Figure 30). Fermentations to which activated charcoal were added fermented to a specific gravity of 1.018 after 192 h whereas fermentations which contained trub fermented to a specific gravity of 1.014 after 145 h. Pure lipid supplements had a stimulative effect over the initial stages of fermentation of brewer's wort (original gravity 1.080) by *Sacch. cerevisiae*. Attenuation of the wort, although initially faster in pure lipid supplemented fermentations, was not significantly greater than the control at the end of the fermentation (1.022 after 192 h compared with 1.024). Fermentations to which trub had been added had attenuated the wort more rapidly and to a greater degree than control, activated charcoal and pure lipid supplemented fermentations (Figures 30 and 31).

FERMENTATION PROFILES SHOWING pH, SPECIFIC GRAVITY AND SUSPENDED YEAST COUNT

The time-course showing pH value was similar in both control and trub-containing fermentations of glucose-salts medium (original gravity 1.060) (Figure 32). The specific gravity drop was more rapid in trub-supplemented fermentations than in the control. A greater number of yeasts were in suspension in trub-supplemented fermentations after 48 h (Figure 33). Results for other supplements, i.e. EDTA-treated trub, EDTA-treated lipid-extracted trub and lipid-extracted trub, were generally similar with all supplements causing a more rapid drop in specific gravity and greater suspended yeast counts than the control (Table 5).

The time-course of pH value was slightly higher in trub-containing fermentations of brewer's wort (original gravity 1.080) than the control but the difference was not significant (Figure 34). A more rapid decrease in specific gravity was recorded in trub-supplemented fermentations in which the suspended yeast counts were higher than in the control (Figure 35). Results for other supplements, i.e. EDTA-treated trub, EDTA-treated lipid-extracted trub and lipid-extracted trub, were

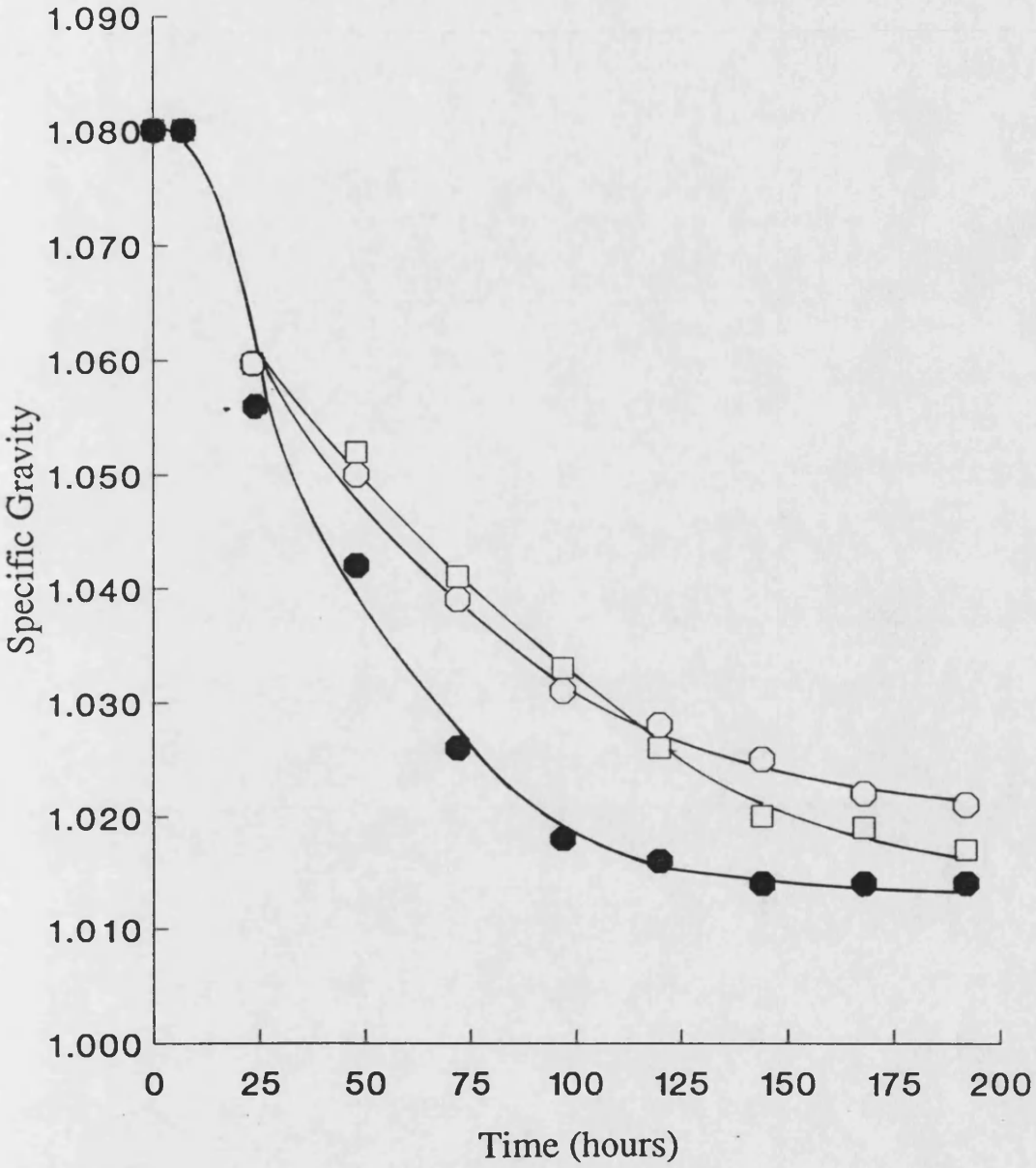


FIGURE 30. Time-course of fermentation of brewer's wort (original gravity 1.080) by *Saccharomyces cerevisiae* showing the effect of neutralised activated charcoal. The values plotted were the average of three independent fermentations and the variation never exceeded the 10% confidence limit. Key: (○) no trub control; (□) 1 g neutralised activated charcoal; (●) 1 g trub.

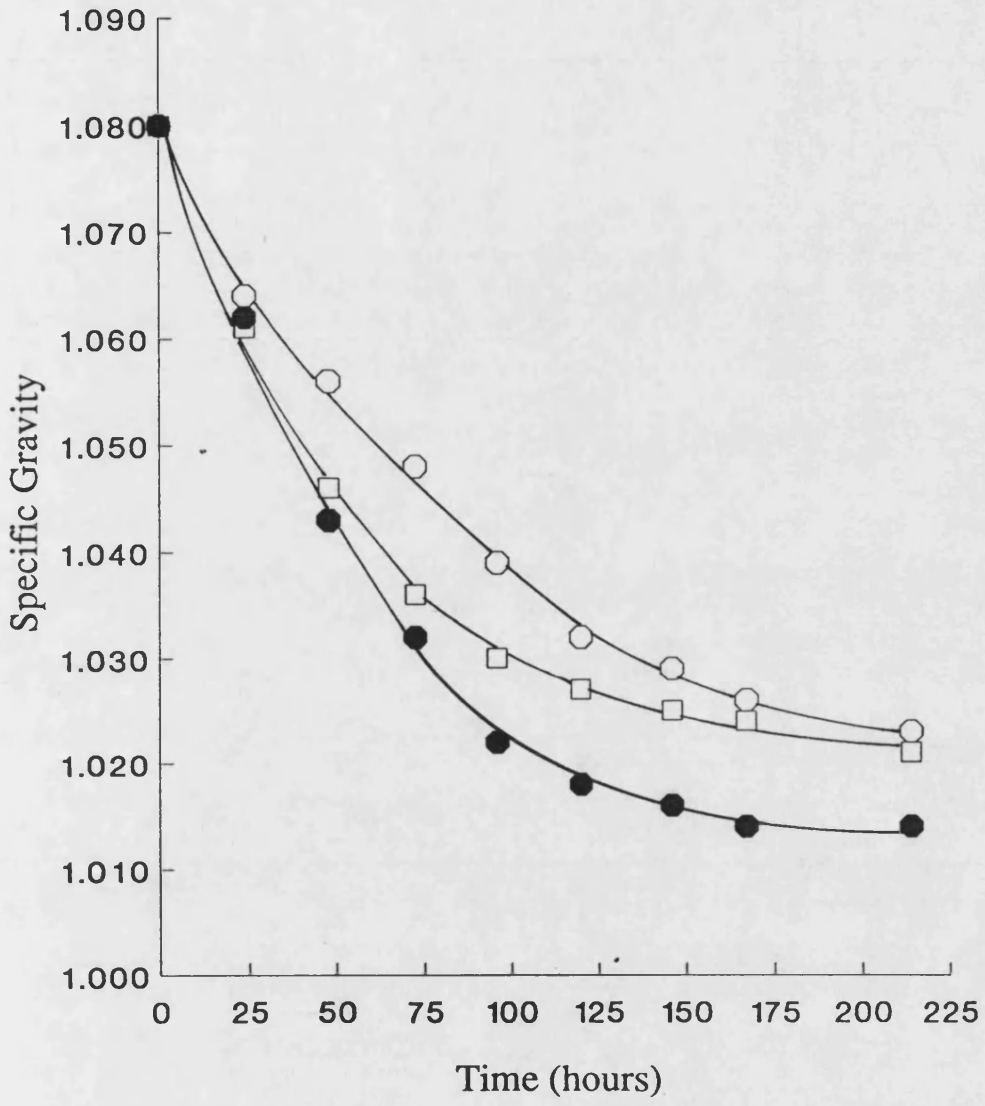


FIGURE 31. Time-course of fermentation of brewer's wort (original gravity 1.080) by *Saccharomyces cerevisiae* showing the effect of pure unsaturated fatty acid and sterol supplements. The unsaturated fatty acid used was linoleic acid and the sterol supplement was β-sitosterol. Both supplements were dissolved in ethanol and added to the wort. The amounts of linoleic acid and β-sitosterol added corresponded to the average amount of each lipid found in trub. The values plotted were the average of three independent fermentations and the variation never exceeded the 10% confidence limit. Key: (○) no trub control; (□) 5.4 mg linoleic acid and 1 mg β-sitosterol; (●) 1 g trub.

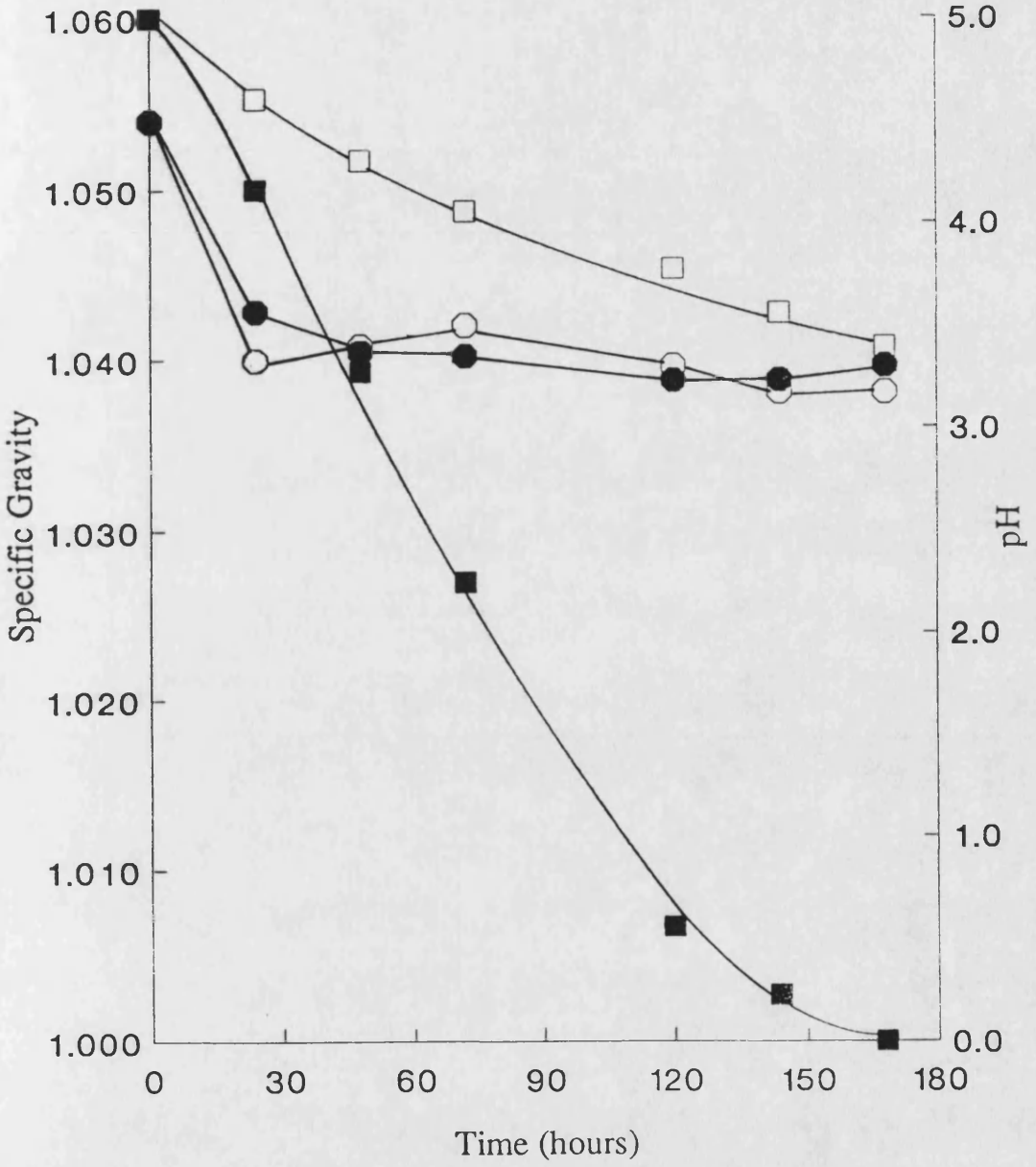


FIGURE 32. Time-course of fermentation of glucose-salts medium (original gravity 1.060) by *Saccharomyces cerevisiae* showing the change in pH (hexagons) and specific gravity (squares). Key: open symbols denote control; closed symbols denote fermentations to which 1 g trub was added. Values plotted were the average of three independent fermentations and the variation never exceeded the 10% confidence limit.

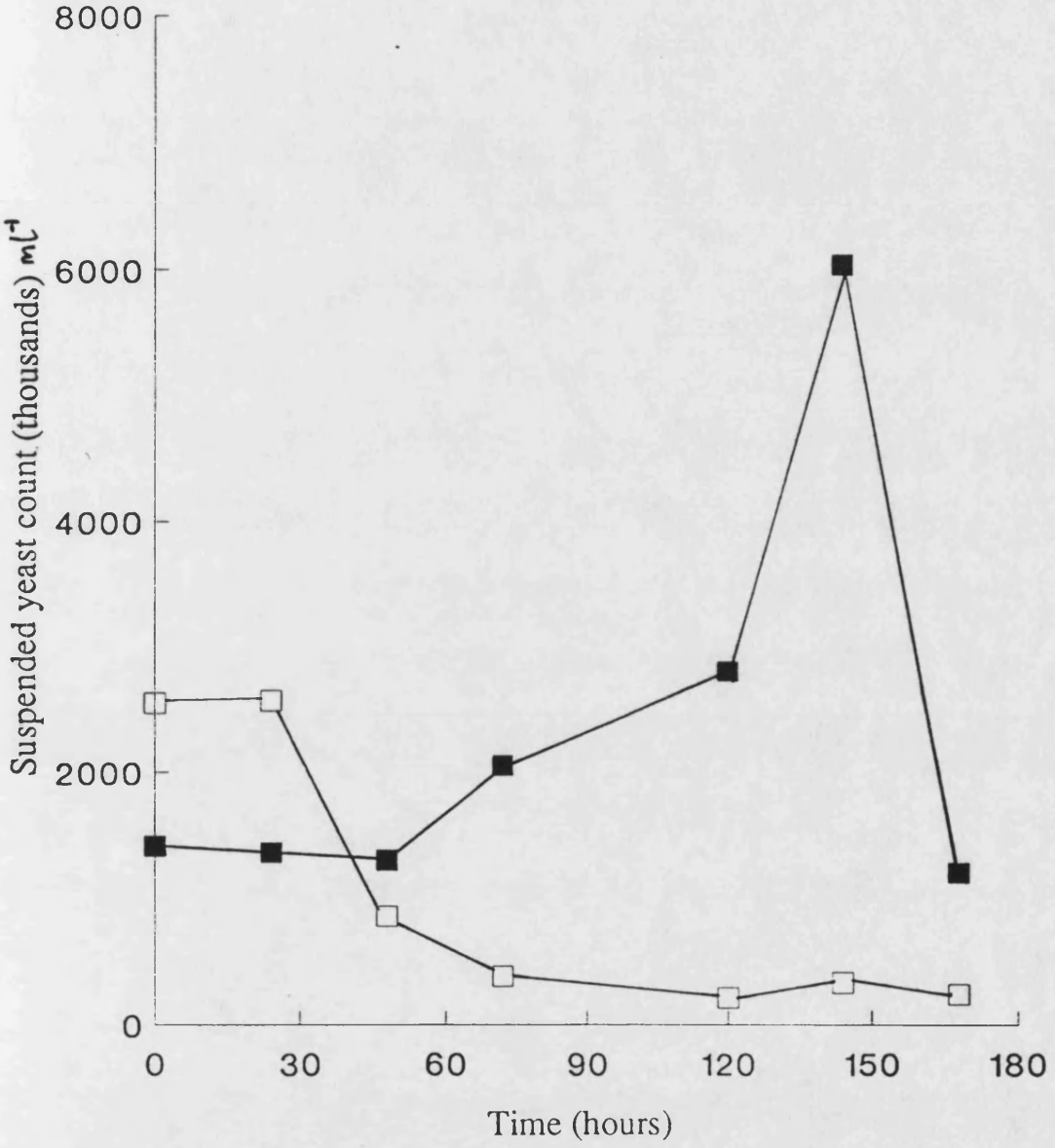


FIGURE 33. Time-course of fermentation of glucose-salts medium (original gravity 1.060) by *Saccharomyces cerevisiae* showing the change in suspended yeast count. Key: open symbols denote control; closed symbols denote fermentations to which 1 g trub was added. Values plotted were the average of three independent fermentations and the variation never exceeded the 10% confidence limit.

TABLE 5. Comparison of specific gravity, pH and suspended yeast cell count after 72 h fermentation of glucose-salts media (original gravity 1.060).

Trub treatment	Specific gravity	pH	Suspended yeast cell count (ml^{-1})
Control[a]	1.049	3.50	3.80×10^5
Trub[a]	1.027	3.35	2.05×10^6
EDTA-treated trub[b]	1.040	3.50	4.20×10^6
Lipid-extracted trub[b]	1.039	3.85	3.62×10^6
EDTA-treated lipid-extracted trub[b]	1.038	3.65	2.24×10^6

[a] values were the average of three independent fermentations

[b] values were the average of two independent fermentations

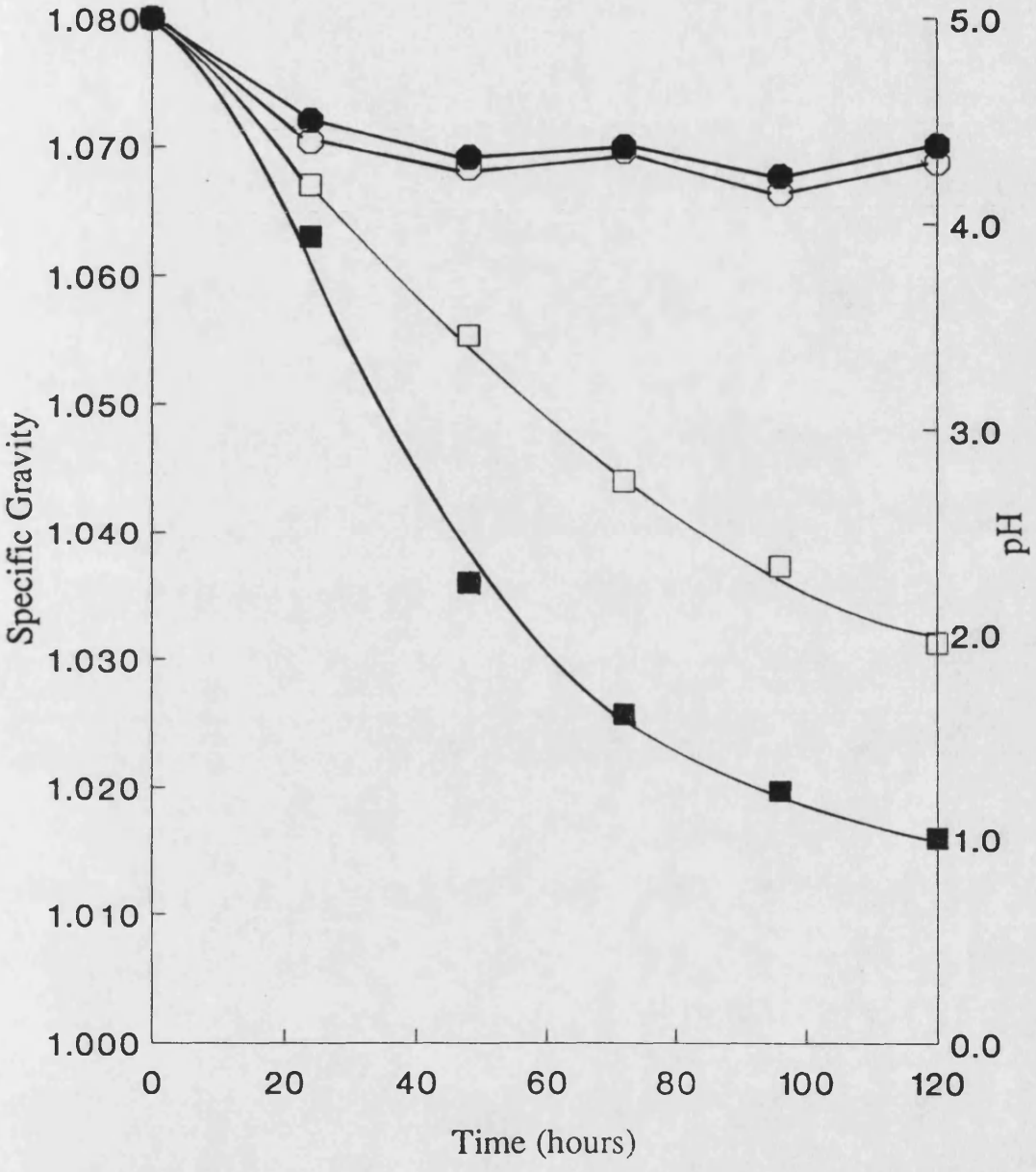


FIGURE 34. Time-course of fermentation of brewer's wort (original gravity 1.080) by *Saccharomyces cerevisiae* showing the change in pH (hexagons) and specific gravity (squares). Key: open symbols denote control; closed symbols denote fermentations to which 1 g trub was added. Values plotted were the average of three independent fermentationsand the variation never exceeded the 10% confidence limit.

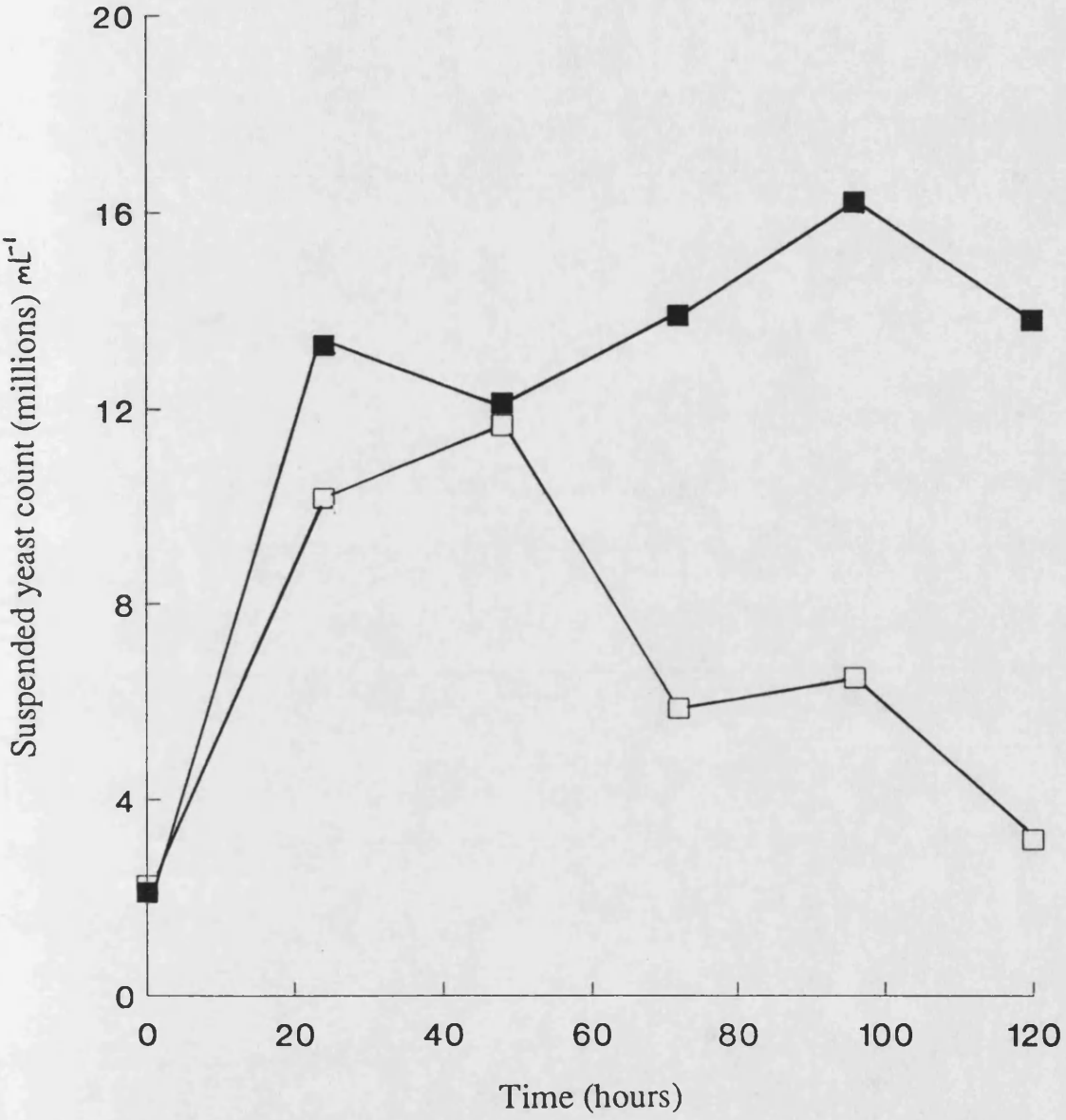


FIGURE 35. Time-course of fermentation of brewer's wort (original gravity 1.080) by *Saccharomyces cerevisiae* showing change in the suspended yeast count. Key: open symbols denote control; closed symbols denote fermentations to which 1 g trub was added. Values plotted were the average of three independent fermentations and the variation never exceeded the 10% confidence limit.

generally similar with all supplements causing a more rapid drop in specific gravity and greater suspended yeast counts than the control (Table 6).

Tables 7 and 8 show greatest yeast growth in trub-supplemented fermentations of both brewer's wort and glucose-salts medium. Yeast growth was greater in control fermentations of glucose-salts medium than in fermentations supplemented with EDTA-treated lipid-extracted trub or lipid-extracted trub. Similarly yeast growth was greater in control fermentations of brewer's wort than in fermentations supplemented with EDTA-treated trub or lipid-extracted trub. For comparison the average inoculum dry weight was 0.042 g per fermentation (the average of six determinations).

TABLE 6. Comparison of specific gravity, pH and suspended yeast cell count after 48 h fermentation of brewer's wort (original gravity 1.080).

Trub treatment	Specific gravity	pH	Suspended yeast cell count (ml^{-1})
Control[a]	1.055	4.30	1.17×10^7
Trub[a]	1.036	4.30	1.21×10^7
EDTA-treated trub[b]	1.046	4.10	1.32×10^7
Lipid-extracted trub[b]	1.053	4.50	6.02×10^6
EDTA-treated lipid-extracted trub[b]	1.046	4.10	1.71×10^7

[a] values were the average of three independent fermentations

[b] values were the average of two independent fermentations

TABLE 7. Yeast dry weight after fermentation of glucose-salt medium (original gravity 1.060).

Trub treatment	Dry weight at end of fermentation (g)
Control[a]	0.148
Trub[a]	0.234
EDTA-treated trub[b]	0.217
Lipid-extracted trub[b]	0.091
EDTA-treated lipid-extracted trub[b]	0.080

[a] values were the average of three independent fermentations

[b] values were the average of two independent fermentations

TABLE 8. Yeast dry weight after fermentation of brewer's wort (original gravity 1.080).

Trub treatment	Dry weight at end of fermentation (g)
Control[a]	0.453
Trub[a]	0.539
EDTA-treated trub[b]	0.407
Lipid-extracted trub[b]	0.309
EDTA-treated lipid-extracted trub[b]	0.468

[a] values were the average of three independent fermentations

[b] values were the average of two independent fermentations

LIPID COMPOSITION OF *SACCHAROMYCES CEREVISIAE* FROM FERMENTATIONS

Glucose-salts fermentations

Saccharomyces cerevisiae harvested from fermentations of glucose-salt media (original gravities 1.060) containing trub was shown to have a different lipid composition compared with the control (Table 9). The principal fatty-acyl residue in total lipid extracts from yeast from both control and trub-supplemented fermentations was $C_{16:0}$ (Table 9). However, differences were apparent with the detection of $C_{18:2}$ and $C_{18:3}$ fatty-acyl residues, and β-sitosterol and stigmasterol in lipid extracts from yeast exposed to trub. These fatty-acyl residues and sterols were largely absent in lipid extracts from control yeast. Values for the total fatty-acyl content, total sterol content and Δ mol^{-1} (the average number of unsaturated bonds per mole; Kates and Hagen, 1964) were greater in trub-containing fermentations. The most abundant unsaturated fatty-acyl in control yeast were $C_{16:1}$ and $C_{18:1}$. Supplementation of glucose-salts medium with linoleic acid and β-sitosterol increased the total fatty-acyl content and the Δ mol^{-1} value in yeast compared to the control, although the Δ mol^{-1} value (0.60) was lower than that for yeast from trub supplemented fermentations (Table 9). Zinc-ion supplementation of glucose-salts medium increased the total fatty-acyl content of yeast but decreased the Δ mol^{-1} value (Table 9). Yeast from fermentations supplemented with lipid-extracted trub showed a lower fatty-acyl content than control yeast, however the Δ mol^{-1} value was greater (Table 10). In yeast from all fermentations $C_{16:0}$ was the most common fatty-acyl residue. Polyunsaturated residues were only present in quantifiable amounts in yeast that had either been exposed to trub or supplemented with linoleic acid and β-sitosterol (Tables 9 and 10). Squalene, a sterol precursor, was abundant in yeast from all fermentations (Tables 11 and 12). The principal sterol present in yeast from all fermentations in Tables 11 and 12 was ergosterol. Non-yeast sterols (ie β-sitosterol and stigmasterol) were only found in yeasts from fermentations supplemented with

TABLE 9. Fatty-acyl composition of *Saccharomyces cerevisiae* harvested from fermentations of glucose-salts medium (specific gravity 1.060) showing the effect of trub, linoleic acid and β–sitosterol supplements and zinc-ion supplement. Values quoted are the average of three independent determinations ± standard deviation.

Fermentation treatment Amount (mg) fatty-acyl residue expressed as $C_{17:0}$ equivalent (g yeast)$^{-1}$				
Fatty-acyl residue	Control	Trub (1 g)	Linoleic acid and β–sitosterol supplements[a]	Zinc-ion supplement[b]
$C_{10:0}$	not detected	trace	2.17 ± 0.13	1.81 ± 0.06
$C_{12:0}$	3.24 ± 0.42	0.48 ± 0.34	3.16 ± 0.13	3.21 ± 0.21
$C_{14:0}$	1.64 ± 0.18	1.19 ± 0.14	2.43 ± 0.13	2.35 ± 0.04
$C_{14:1}$	0.42 ± 0.06	trace	trace	0.37 ± 0.05
$C_{16:0}$	6.64 ± 0.32	6.85 ± 0.58	9.60 ± 0.35	8.98 ± 0.20
$C_{16:1}$	4.82 ± 0.41	2.56 ± 0.23	4.03 ± 0.29	5.96 ± 0.16
$C_{18:0}$	2.34 ± 0.26	3.24 ± 0.36	3.83 ± 0.13	3.40 ± 0.08
$C_{18:1}$	3.42 ± 0.12	4.03 ± 0.56	4.41 ± 0.14	5.77 ± 0.46
$C_{18:2}$	not detected	6.77 ± 0.41	6.77 ± 0.89	trace
$C_{18:3}$	not detected	1.15 ± 0.04	trace	not detected
Total	22.52	26.27	36.40	31.85
Δ mol^{-1}	0.39	0.9	0.60	0.38

[a] 5.4 mg linoleic acid and 1 mg β–sitosterol (200 ml medium)$^{-1}$

[b] 128 μg $ZnSO_4$ (200 ml medium)$^{-1}$

TABLE 10. Fatty-acyl composition of *Saccharomyces cerevisiae* harvested from fermentations of glucose-salts medium (specific gravity 1.060) showing the effect of lipid-extracted trub. Control and trub values are the same as in Table 9. Values quoted are the average of three independent determinations ± standard deviation.

	Fermentation treatment Amount (mg) fatty-acyl residue expressed as $C_{17:0}$ equivalent (g yeast)$^{-1}$		
Fatty-acyl residue	Control	Trub (1 g)	Lipid-extracted trub (1 g)
$C_{10:0}$	not detected	trace	0.17 ± 0.02
$C_{12:0}$	3.24 ± 0.42	0.48 ± 0.34	0.30 ± 0.02
$C_{14:0}$	1.64 ± 0.18	1.19 ± 0.14	0.38 ± 0.06
$C_{14:1}$	0.42 ± 0.06	trace	trace
$C_{16:0}$	6.64 ± 0.32	6.85 ± 0.58	3.20 ± 0.27
$C_{16:1}$	4.82 ± 0.41	2.56 ± 0.23	2.86 ± 0.11
$C_{18:0}$	2.34 ± 0.26	3.24 ± 0.36	2.68 ± 0.40
$C_{18:1}$	3.42 ± 0.12	4.03 ± 0.56	2.66 ± 0.55
$C_{18:2}$	not detected	6.77 ± 0.41	trace
$C_{18:3}$	not detected	1.15 ± 0.04	trace
Total	22.52	26.27	26.31
Δ mol^{-1}	0.39	0.9	0.43

TABLE 11. Sterol composition of *Saccharomyces cerevisiae* harvested from fermentations of glucose-salts medium (specific gravity 1.060) showing the effect of trub, linoleic acid and β–sitosterol supplements and zinc-ion supplement. Values quoted are the average of three independent determinations ± standard deviation.

	Fermentation treatment Amount (mg) sterol expressed as cholestanol equivalent (g yeast)$^{-1}$			
Sterol or precursor	Control	Trub (1 g)	Linoleic acid and β-sitosterol supplements[a]	Zinc-ion supplement[b]
Squalene	4.76 ± 0.30	2.28 ± 0.20	10.60 ± 0.40	11.08 ± 0.25
Ergosterol	0.24 ± 0.10	1.38 ± 0.14	1.89 ± 0.1	2.28 ± 0.08
Lanosterol	0.08 ± 0.01	0.11 ± 0.01	trace	trace
β-Sitosterol	not detected	0.52 ± 0.03	1.12 ± 0.11	not detected
Stigmasterol	trace	0.49 ± 0.04	0.39 ± 0.10	trace
Total sterol	0.32	2.5	3.4	2.28

[a] 5.4 mg linoleic acid and 1 mg β–sitosterol (200 ml medium)$^{-1}$

[b] 128 μg $ZnSO_4$ (200 ml medium)$^{-1}$

TABLE 12. Sterol composition of *Saccharomyces cerevisiae* harvested from fermentations of glucose-salts medium (specific gravity 1.060) showing the effect of lipid-extracted trub. Control and trub values are the same as in Table 11. Values quoted are the average of three independent determinations ± standard deviation.

	Fermentation treatment Amount (mg) sterol expressed as cholestanol equivalent (g yeast)$^{-1}$		
Sterol or precursor	Control	Trub (1 g)	Lipid-extracted trub (1 g)
Squalene	4.76 ± 0.30	2.28 ± 0.20	8.72 ± 0.61
Ergosterol	0.24 ± 0.10	1.38 ± 0.14	1.81 ± 0.11
Lanosterol	0.08 ± 0.01	0.11 ± 0.01	0.16 ± 0.05
β-Sitosterol	not detected	0.52 ± 0.03	trace
Stigmasterol	trace	0.49 ± 0.04	not detected
Total sterol	0.32	2.5	1.97

either trub or linoleic acid and β-sitosterol. The total sterol content in yeast from control fermentations was considerably lower than in yeast from other fermentations (Tables 11 and 12). The Δ mol^{-1} values for yeast from fermentations supplemented with trub lipid extract and EDTA-treated trub were greater than in the control (Tables 11 and 12). Futhermore, the Δ mol^{-1} value of yeast from fermentations supplemented with trub lipid extract was greater than that for yeast from trub-supplemented fermentations (Table 13). However, the Δ mol^{-1} value for yeast from fermentations supplemented with ashed trub was slightly lower than that of the control (Table 13). Polyunsaturated fatty-acyl residues were only found in quantifiable amounts in yeasts from fermentations supplemented with EDTA-treated trub, trub lipid extract or trub (Tables 13 and 14). Ergosterol was the principal sterol found in yeast all fermentations of glucose-salts medium in Tables 15 and 16. β-Sitosterol and stigmasterol were found in yeast from fermentations supplemented with EDTA-treated trub, trub lipid extract and trub but not in yeast from control or ashed trub-supplemented fermentations.

Yeast from trub-supplemented fermentations at specific gravity 1.080 had a greater total fatty-acyl content and Δ mol^{-1} value than than those from control fermentations (Table 17). Polyunsaturated fatty-acyl residues were only found in quantifiable amounts in yeast from trub-supplemented fermentations.

Brewer's wort

The major fatty-acyl residue in *Saccharomyces cerevisiae* from fermentations of brewer's wort (specific gravity 1.080) was $C_{16:0}$ (Table 18). The Δ mol^{-1} value was greatest in yeast from trub-supplemented fermentations. In yeast from fermentations supplemented with ashed trub the Δ mol^{-1} value was lower than that of the control. Yeast from fermentations supplemented with trub or ashed trub had lower total fatty-acyl contents than control yeast. However, yeast from fermentations supplemented with the soluble fraction of ashed trub showed a greater fatty-acyl content and Δ mol^{-1} value than control yeast. Sterol analysis revealed that β-sitosterol was most common

TABLE 13. Fatty-acyl composition of *Saccharomyces cerevisiae* harvested from fermentations of glucose-salts medium (specific gravity 1.060) showing the effect of ashed trub and trub lipid extract. Values quoted are the average of three independent determinations ± standard deviation.

	Fermentation treatment Amount (mg) fatty-acyl residue expressed as $C_{17:0}$ equivalent (g yeast)$^{-1}$			
Fatty-acyl residue	Control	Trub lipid extract (1 ml)	Ashed trub trub (1 g)	Trub (1g)
$C_{10:0}$	not detected	trace	1.38 ± 0.10	trace
$C_{12:0}$	1.07 ± 0.03	trace	2.74 ± 0.19	0.20 ± 0.03
$C_{14:0}$	0.88 ± 0.08	1.29 ± 0.18	1.57 ± 0.09	0.43 ± 0.03
$C_{14:1}$	0.15 ± 0.01	trace	trace	trace
$C_{16:0}$	4.51 ± 0.20	9.04 ± 0.74	6.60 ± 0.47	4.76 ± 0.04
$C_{16:1}$	3.25 ± 0.05	4.81 ± 0.41	5.34 ± 0.47	2.85 ± 0.07
$C_{18:0}$	1.87 ± 0.06	2.53 ± 0.26	2.73 ± 0.13	2.62 ± 0.04
$C_{18:1}$	3.96 ± 0.02	7.08 ± 0.64	5.65 ± 0.46	2.79 ± 0.03
$C_{18:2}$	not detected	4.05 ± 0.37	trace	2.07 ± 0.03
$C_{18:3}$	not detected	trace	not detected	0.16 ± 0.04
Total	15.69	28.8	26.31	15.88
Δ mol^{-1}	0.47	0.69	0.43	0.65

TABLE 14. Fatty-acyl composition of *Saccharomyces cerevisiae* harvested from fermentations of glucose-salts medium (specific gravity 1.060) showing the effect of EDTA-treated trub. Control and trub values are the same as in Table 13. Values quoted are the average of three independent determinations ± standard deviation.

	Fermentation treatment Amount (mg) fatty-acyl residue expressed as $C_{17:0}$ equivalent (g yeast)$^{-1}$		
Fatty-acyl residue	Control	Trub (1 g)	EDTA-treated trub (1 g)
$C_{10:0}$	not detected	trace	trace
$C_{12:0}$	1.07 ± 0.03	0.20 ± 0.03	0.25 ± 0.04
$C_{14:0}$	0.88 ± 0.08	0.43 ± 0.03	0.83 ± 0.33
$C_{14:1}$	0.15 ± 0.01	trace	trace
$C_{16:0}$	4.51 ± 0.20	4.76 ± 0.04	4.70 ± 0.55
$C_{16:1}$	3.25 ± 0.05	2.85 ± 0.07	2.31 ± 0.38
$C_{18:0}$	1.87 ± 0.06	2.62 ± 0.04	2.03 ± 0.33
$C_{18:1}$	3.96 ± 0.02	2.79 ± 0.03	3.50 ± 0.44
$C_{18:2}$	not detected	2.07 ± 0.03	1.70 ± 0.04
$C_{18:3}$	not detected	0.16 ± 0.04	0.20 ± 0.05
Total	15.69	15.72	15.22
Δ mol^{-1}	0.47	0.65	0.63

TABLE 15. Sterol composition of *Saccharomyces cerevisiae* harvested from fermentations of glucose-salts medium (specific gravity 1.060) showing the effect of EDTA-treated trub and the lipid extract of trub. Values quoted are the average of three independent determinations ± standard deviation.

	Fermentation treatment Amount (mg) sterol expressed as cholestanol equivalents (g yeast)$^{-1}$			
Sterol or precursor	Control	Trub (1 g)	EDTA-Treated trub (1 g)	Trub lipid extract (1 ml)
Squalene	7.61 ± 0.11	3.29 ± 0.17	11.40 ± 0.72	10.26 ± 0.71
Ergosterol	0.81 ± 0.04	1.79 ± 0.08	0.91 ± 0.09	1.86 ± 0.15
Lanosterol	0.15 ± 0.02	0.13 ± 0.02	0.57 ± 0.10	trace
β-Sitosterol	not detected	0.46 ± 0.03	0.41 ± 0.06	0.92 ± 0.15
Stigmasterol	not detected	0.28 ± 0.02	0.22 ± 0.04	0.16 ± 0.02
Total sterol	0.96	2.66	2.11	2.9

TABLE 16. Sterol composition of *Saccharomyces cerevisiae* harvested from fermentations of glucose-salts medium (specific gravity 1.060) showing the effect of ashed trub. Control and trub values are the same as in Table 15. Values quoted are the average of three independent determinations ± standard deviation

	Fermentation treatment Amount (mg) sterol expressed as cholestanol equivalents (g yeast)$^{-1}$		
Sterol or precursor	Control	Trub (1 g)	Ashed trub (1 g)
Squalene	7.61 ± 0.11	3.29 ± 0.17	9.53 ± 0.05
Ergosterol	0.81 ± 0.04	1.79 ± 0.08	2.23 ± 0.05
Lanosterol	0.15 ± 0.02	0.13 ± 0.02	trace
β-Sitosterol	not detected	0.46 ± 0.03	not detected
Stigmasterol	not detected	0.28 ± 0.02	0.13 ± 0.02
Total sterol	0.96	2.66	2.36

TABLE 17. Fatty-acyl composition of *Saccharomyces cerevisiae* harvested from fermentations of glucose-salts medium (specific gravity 1.080) showing the effect of trub. Values quoted are the average of three independent determinations ± standard deviation.

Fermentation treatment Amount (mg) fatty-acyl residue expressed as $C_{17:0}$ equivalent (g yeast)$^{-1}$		
Fatty-acyl residue	Control	Trub (1 g)
$C_{10:0}$	0.96 ± 0.09	1.02 ± 0.05
$C_{12:0}$	2.31 ± 0.26	1.98 ± 0.05
$C_{14:0}$	0.64 ± 0.08	0.83 ± 0.06
$C_{14:1}$	trace	trace
$C_{16:0}$	7.88 ± 0.41	9.58 ± 0.61
$C_{16:1}$	1.81 ± 0.12	2.94 ± 0.39
$C_{18:0}$	3.49 ± 0.52	5.19 ± 0.27
$C_{18:1}$	1.25 ± 0.14	2.47 ± 0.34
$C_{18:2}$	trace	2.66 ± 0.39
$C_{18:3}$	not detected	trace
Total	18.34	24.67
Δ mol[1]	0.17	0.43

TABLE 18. Fatty-acyl composition of *Saccharomyces cerevisiae* harvested from fermentations of brewer's wort (specific gravity 1.080) showing the effect of trub, ashed trub and the soluble fraction of ashed trub. Values quoted are the average of three independent determinations ± standard deviation.

Fermentation treatment and specific gravity Amount (mg) fatty-acyl residue expressed as $C_{17:0}$ equivalent (g yeast)$^{-1}$				
Fatty-acyl residue	Control	Trub (1 g)	Ashed trub (1 g)	Soluble fraction of ashed trub (10 ml)
$C_{10:0}$	not detected	not detected	2.81 ± 0.37	1.39 ± 0.06
$C_{12:0}$	1.47 ± 0.16	3.74 ± 0.23	3.20 ± 0.32	3.36 ± 0.70
$C_{14:0}$	0.99 ± 0.12	0.73 ± 0.11	0.86 ± 0.09	1.48 ± 0.69
$C_{14:1}$	trace	trace	trace	trace
$C_{16:0}$	12.00 ± 0.81	6.75 ± 0.21	8.19 ± 0.17	14.30 ± 0.11
$C_{16:1}$	4.20 ± 0.25	3.02 ± 0.17	3.18 ± 0.15	3.03 ± 0.30
$C_{18:0}$	5.31 ± 0.36	3.09 ± 0.12	4.59 ± 0.27	7.59 ± 0.21
$C_{18:1}$	3.32 ± 0.23	2.67 ± 0.21	2.00 ± 0.18	11.33 ± 0.81
$C_{18:2}$	2.70 ± 0.15	5.24 ± 0.34	1.28 ± 0.12	5.48 ± 0.47
$C_{18:3}$	0.16 ± 0.06	0.28 ± 0.12	0.41 ± 0.10	0.15 ± 0.03
Total	32.32	23.25	26.32	34.79
Δ mol^{-1}	0.41	0.73	0.32	0.47

in yeast from control and trub-supplemented fermentations but was absent from yeast exposed to ashed trub or the soluble fraction of ashed trub. In yeast from these fermentations lanosterol was the most common sterol (Table 19).

Yeast from fermentations of turbid brewer's wort or wort (specific gravity 1.080) supplemented with EDTA-treated lipid-extracted trub or linoleic acid and β-sitosterol had greater total fatty-acyl contents and Δ mol^{-1} values than yeast from the corresponding control and trub-supplemented fermentations (Tables 20 and 21). Ergosterol was the most abundant sterol in yeast from all fermentations except those supplemented with linoleic acid and β-sitosterol (Tables 22 and 23). Yeast from fermentations of brewer's wort (specific gravity 1.060) supplemented with lipid-extracted trub had a lower Δ mol^{-1} value than yeast from control or trub-supplemented fermentations (Table 24). The most abundant sterol in yeasts from fermentations supplemented with lipid-extracted trub was β-sitosterol with ergosterol also prominent (Table 25). The ranking of these two sterols was reversed in yeast from control and trub-supplemented fermentations with ergosterol being the principal sterol. Yeast from fermentations of brewer's wort (specific gravity 1.100) had a greater Δ mol^{-1} value than yeast from control fermentations (Table 26). The total sterol contents were almost identical with β-sitosterol the most abundant in yeast from both control and trub-supplemented fermentations (Table 27).

In summary $C_{16:0}$ was the most abundant fatty-acyl residue in yeast regardless of the fermentation treatment. Yeast from fermentations to which trub, trub lipid extract, EDTA-treated trub or β–sitosterol and linoleic acid were added displayed high Δ mol^{-1} values compared to yeast from control fermentations or fermentations supplemented with lipid-extracted trub, ashed trub, soluble fraction of ashed trub or zinc ions. Large amounts of squalene were present in all yeast regardless of their origin. β-Sitosterol and stigmasterol were found in lipid extracts from yeast from fermentations of glucose-salts medium supplemented with trub, EDTA-treated trub, trub lipid extract and β–sitosterol and linoleic acid, whereas they were absent in lipid

TABLE 19. Sterol composition of *Saccharomyces cerevisiae* harvested from fermentations of brewer's wort (specific gravity 1.080) showing the effect of trub, ashed trub and the soluble fraction of ashed trub. Values quoted are the average of three independent determinations ± standard deviation.

	Fermentation treatment Amount (mg) sterol expressed as cholestanol equivalent (g yeast)$^{-1}$			
Sterol or precursor	Control	Trub (1 g)	Ashed trub (1 g)	Soluble fraction of ashed trub (10 ml)
Squalene	29.4 ± 0.95	22.6 ± 0.72	23.42 ± 1.20	22.79 ± 1.08
Ergosterol	0.26 ± 0.08	0.82 ± 0.13	0.31 ± 0.08	0.53 ± 0.08
Lanosterol	0.15 ± 0.03	0.16 ± 0.05	0.43 ± 0.04	0.81 ± 0.05
β–Sitosterol	1.08 ± 0.22	1.40 ± 0.25	trace	not detected
Stigmasterol	trace	trace	not detected	not detected
Total sterol	1.49	2.38	1.49	1.52

TABLE 20. Fatty-acyl composition of *Saccharomyces cerevisiae* harvested from fermentations of brewer's wort (specific gravity 1.080) showing the effect of EDTA-treated lipid-extracted trub and linoleic acid and β-sitosterol supplements. Values quoted are the average of three independent determinations ± standard deviation.

Fermentation treatment Amount (mg) fatty-acyl residue expressed as $C_{17:0}$ equivalent (g yeast)$^{-1}$				
Fatty-acyl residue	Control	EDTA-Treated lipid-extracted trub (1 g)	Linoleic acid and β-Sitosterol supplements[a]	Trub (1 g)
$C_{10:0}$	0.96 ± 0.09	not detected	3.27 ± 0.56	1.02 ± 0.03
$C_{12:0}$	2.31 ± 0.20	2.51 ± 0.61	3.91 ± 0.63	1.98 ± 0.03
$C_{14:0}$	0.64 ± 0.08	trace	2.48 ± 0.04	0.83 ± 0.02
$C_{14:1}$	trace	trace	trace	trace
$C_{16:0}$	7.88 ± 0.35	5.62 ± 0.74	17.15 ± 0.75	7.58 ± 0.27
$C_{16:1}$	1.81 ± 0.12	2.65 ± 0.12	3.06 ± 0.25	2.94 ± 0.19
$C_{18:0}$	3.49 ± 0.12	3.34 ± 0.33	7.63 ± 0.25	5.19 ± 0.17
$C_{18:1}$	1.25 ± 0.14	3.05 ± 0.48	14.25 ± 0.87	2.47 ± 0.04
$C_{18:2}$	2.15 ± 0.10	3.82 ± 0.28	12.78 ± 0.40	4.66 ± 0.09
$C_{18:3}$	not detected	trace	0.26 ± 0.05	trace
Total	18.34	20.99	64.79	26.67
Δ mol^{-1}	0.40	0.64	0.67	0.55

[a] 5.4 mg linoleic acid and 1 mg β–sitosterol (200 ml medium)$^{-1}$

TABLE 21. Fatty-acyl composition of *Saccharomyces cerevisiae* harvested from fermentations of brewer's wort (specific gravity 1.080) showing the effect of turbid wort. Control and trub values are the same as in Table 20. Values quoted are the average of three independent determinations ± standard deviation

Fermentation treatment Amount (mg) fatty-acyl residue expressed as $C_{17:0}$ equivalent (g yeast)$^{-1}$			
Fatty-acyl residue	Control	Trub (1 g)	Turbid wort
$C_{10:0}$	0.96 ± 0.09	1.02 ± 0.03	not detected
$C_{12:0}$	2.31 ± 0.20	1.98 ± 0.03	3.34 ± 0.31
$C_{14:0}$	0.64 ± 0.08	0.83 ± 0.02	0.74 ± 0.06
$C_{14:1}$	trace	trace	0.15 ± 0.05
$C_{16:0}$	7.88 ± 0.35	7.58 ± 0.27	8.83 ± 0.31
$C_{16:1}$	1.81 ± 0.12	2.94 ± 0.19	5.56 ± 0.27
$C_{18:0}$	3.49 ± 0.12	5.19 ± 0.17	4.45 ± 0.38
$C_{18:1}$	1.25 ± 0.14	2.47 ± 0.04	4.21 ± 0.21
$C_{18:2}$	2.15 ± 0.10	4.66 ± 0.09	4.69 ± 0.36
$C_{18:3}$	not detected	trace	0.72 ± 0.21
Total	18.34	26.67	32.69
Δ mol^{-1}	0.40	0.55	0.65

TABLE 22. Sterol composition of *Saccharomyces cerevisiae* harvested from fermentations of brewer's wort (specific gravity 1.080) showing the effect of EDTA-treated lipid-extracted trub and linoleic acid and β-sitosterol supplements. Values quoted are the average of three independent determinations ± standard deviation.

	Fermentation treatment Amount (mg) sterol expressed as cholestanol equivalent (g yeast)$^{-1}$			
Sterol or precursor	Control (1 g)	EDTA-Treated lipid-extracted trub (1 g)	Linoleic acid and β-Sitosterol supplements[a]	Trub (1 g)
Squalene	19.00 ± 0.32	14.69 ± 0.84	22.46 ± 1.23	17.43 ± 0.42
Ergosterol	0.88 ± 0.06	0.52 ± 0.11	0.42 ± 0.06	0.75 ± 0.05
Lanosterol	0.09 ± 0.02	0.43 ± 0.09	1.03 ± 0.10	0.59 ± 0.08
β–Sitosterol	0.08 ± 0.03	0.16 ± 0.02	0.99 ± 0.11	0.61 ± 0.07
Stigmasterol	not detected	not detected	not detected	not detected
Total sterol	1.05	2.38	2.53	1.95

[a] 5.4 mg linoleic acid and 1 mg β–sitosterol (200 ml medium)$^{-1}$

TABLE 23. Sterol composition of *Saccharomyces cerevisiae* harvested from fermentations of brewer's wort (specifc gravity 1.080) showing the effect of turbid wort. Control and trub values are the same as in Table 22. Values quoted are the average of three independent determinations ± standard deviation.

	Fermentation treatment and specific gravity Amount (mg) sterol expressed as cholestanol equivalent (g yeast)$^{-1}$		
Sterol or precursor	Control	Turbid wort	Trub (1 g)
Squalene	19.00 ± 0.32	22.76 ± 1.16	17.43 ± 0.42
Ergosterol	0.88 ± 0.06	0.86 ± 0.15	0.75 ± 0.05
Lanosterol	0.09 ± 0.02	0.80 ± 0.24	0.59 ± 0.08
β-Sitosterol	0.08 ± 0.03	0.36 ± 0.12	0.61 ± 0.07
Stigmasterol	not detected	not detected	not detected
Total sterol	1.05	2.02	1.95

TABLE 24. Fatty-acyl composition of *Saccharomyces cerevisiae* harvested from fermentations of brewer's wort (specific gravity 1.060) showing the effect of lipid-extracted trub. Values quoted are the average of three independent determinations ± standard deviation.

	Fermentation treatment Amount (mg) fatty-acyl residue expressed as $C_{17:0}$ equivalent (g yeast)$^{-1}$		
Fatty-acyl residue	Control	Trub (1 g)	Lipid-extracted trub (1 g)
$C_{10:0}$	not detected	not detected	1.74 ± 0.34
$C_{12:0}$	1.01 ± 0.05	1.74 ± 0.23	1.82 ± 0.23
$C_{14:0}$	0.43 ± 0.02	0.80 ± 0.11	1.29 ± 0.15
$C_{14:1}$	not detected	trace	trace
$C_{16:0}$	6.31 ± 0.15	5.59 ± 0.24	6.59 ± 0.12
$C_{16:1}$	1.51 ± 0.06	1.24 ± 0.05	1.71 ± 0.10
$C_{18:0}$	3.26 ± 0.11	3.61 ± 0.17	2.25 ± 0.15
$C_{18:1}$	2.44 ± 0.17	3.11 ± 0.11	1.51 ± 0.16
$C_{18:2}$	2.56 ± 0.18	4.12 ± 0.13	2.63 ± 0.28
$C_{18:3}$	0.14 ± 0.02	0.51 ± 0.06	0.12 ± 0.05
Total	17.66	20.72	19.93
Δ mol^{-1}	0.54	0.68	0..44

TABLE 25. Sterol composition of *Saccharomyces cerevisiae* harvested from fermentations of brewer's wort (specific gravity 1.060) showing the effect of lipid-extracted trub. Values quoted are the average of three independent determinations ± standard deviation.

	Fermentation treatment Amount (mg) sterol expressed as cholestanol equivalent (g yeast)$^{-1}$		
Sterol or precursor	Control	Trub (1 g)	lipid-extracted trub (1 g)
Squalene	8.11 ± 0.24	4.66 ± 0.31	7.86 ± 0.05
Ergosterol	0.52 ± 0.08	0.89 ± 0.06	0.56 ± 0.17
Lanosterol	0.37 ± 0.02	0.25 ± 0.04	0.27 ± 0.05
β–Sitosterol	0.81 ± 0.04	0.72 ± 0.07	0.63 ± 0.15
Stigmasterol	not detected	0.11 ± 0.03	0.35 ± 0.06
Total sterol	1.60	1.97	1.51

TABLE 26. Fatty-acyl composition of *Saccharomyces cerevisiae* harvested from fermentations of brewer's wort (specific gravity 1.100) showing the effect of trub. Values quoted are the average of three independent determinations ± standard deviation.

Fermentation treatment Amount (mg) fatty-acyl residue expressed as $C_{17:0}$ equivalent (g yeast)$^{-1}$		
Fatty-acyl residue	Control	Trub (1 g)
$C_{10:0}$	0.65 ± 0.10	not detected
$C_{12:0}$	1.86 ± 0.26	1.58 ± 0.11
$C_{14:0}$	1.26 ± 0.11	0.86 ± 0.05
$C_{14:1}$	trace	trace
$C_{16:0}$	10.32 ± 0.21	8.57 ± 0.82
$C_{16:1}$	2.06 ± 0.10	3.30 ± 0.15
$C_{18:0}$	4.92 ± 0.17	4.21 ± 0.18
$C_{18:1}$	2.44 ± 0.37	3.03 ± 0.17
$C_{18:2}$	1.59 ± 0.16	3.69 ± 0.26
$C_{18:3}$	0.29 ± 0.02	0.24 ± 0.05
Total	25.39	25.48
Δ mol^{-1}	0.34	0.57

TABLE 27. Sterol composition of *Saccharomyces cerevisiae* harvested from fermentations of brewer's wort (specific gravity 1.100) showing the effect of trub. Values quoted are the average of three independent determinations ± standard deviation.

	Fermentation treatment Amount (mg) sterol expressed as cholestanol equivalent (g yeast)$^{-1}$	
Sterol or precursor	Control	Trub (1 g)
Squalene	19.4 ± 0.45	20.86 ± 0.56
Ergosterol	0.38 ± 0.06	0.24 ± 0.04
Lanosterol	trace	0.58 ± 0.08
β–Sitosterol	1.13 ± 0.22	0.70 ± 0.09
Stigmasterol	trace	trace
Total sterol	1.51	1.52

extracts from yeast from control fermentations and fermentations supplemented with ashed trub, lipid-extracted trub and zinc ions.

LIPID COMPOSITION OF STARTER YEAST

Starter cultures of *Saccharomyces cerevisiae* were harvested, washed and freeze-dried as already described in the Methods section. Lipids were extracted from 250 mg freeze-dried starter yeast and analysed as already described in the Methods section. Starter yeast was found to be rich in monounsaturated fatty-acyl residues, $C_{16:1}$ and $C_{18:1}$ (Table 28). The Δ mol^{-1} value was 0.80 . Ergosterol was the principal sterol with a small quantity of lanosterol and a trace of stigmasterol present (Table 28).

LIPID COMPOSITION OF GLUCOSE-SALTS MEDIUM

Portions (50 ml) of glucose-salts medium (original gravity 1.060) were frozen overnight at -20°C and freeze-dried as already described in the Methods section. Lipids were extracted and analysed as already described in the Methods section. The principal fatty-acyl residues in the medium were $C_{16:0}$ and $C_{18:0}$, respectively (Table 29). Only unsaturated residue present in a quantifiable amount was $C_{18:1}$. No sterols were detected.

TABLE 28. Fatty-acyl and sterol composition of *Saccharomyces cerevisiae* harvested from aerobic starter cultures of glucose salts medium (original gravity 1.060). Values quoted are the average of three independent determinations ± standard deviation.

Fatty-acyl residue	Amount (mg) fatty-acyl residue expressed as $C_{17:0}$ equivalent $(g\ yeast)^{-1}$	Sterol or precursor	Amount (mg) sterol expressed as cholestanol equivalent $(g\ yeast)^{-1}$
$C_{10:0}$	not detected	Squalene	1.12 ± 0.03
$C_{12:0}$	0.22 ± 0.04		
$C_{14:0}$	0.70 ± 0.11	Ergosterol	11.70 ± 0.36
$C_{14:1}$	0.24 ± 0.03		
$C_{16:0}$	6.70 ± 0.48	Lanosterol	0.36 ± 0.03
$C_{16:1}$	18.92 ± 0.91		
$C_{18:0}$	2.37 ± 0.21	β-Sitosterol	not detected
$C_{18:1}$	22.42 ± 1.12		
$C_{18:2}$	not detected	Stigmasterol	trace
$C_{18:3}$	not detected		
Total	51.57		12.06
$\Delta\ mol^{-1}$	0.80		-

TABLE 29. Fatty-acyl composition of glucose salts medium (original gravity 1.060). Values quoted are the average of three independent determinations ± standard deviation.

Fatty-acyl residue	Amount (mg) fatty-acyl residue expressed as $C_{17:0}$ equivalent (l medium)$^{-1}$
$C_{12:0}$	trace
$C_{14:0}$	trace
$C_{14:1}$	not detected
$C_{16:0}$	3.39 ± 0.56
$C_{16:1}$	trace
$C_{18:0}$	4.03 ± 0.88
$C_{18:1}$	1.10 ± 0.25
$C_{18:2}$	not detected
$C_{18:3}$	not detected
Total	8.52

EFFECT OF FERMENTATION TREATMENTS ON THE MAGNESIUM AND ZINC CONTENT OF *SACCHAROMYCES CEREVISIAE*

The average amount of zinc present in yeast from ash- and trub-supplemented fermentations of glucose-salts medium (original gravity 1.060) was not significantly different ($p = 0.05$) from the average for zinc found in the control yeast (Table 30). However, the mean zinc content of yeast from fermentations of the same medium, supplemented with either linoleic acid and β-sitosterol or zinc ions, differed significantly ($p = 0.05$) from the mean found in the control. The average amount of magnesium in yeast from supplemented fermentations did not differ significantly ($p = 0.05$) from that found in the control yeast except in the case of yeast from ash-supplemented fermentations, where the magnesium content (2.2 mg) was significantly greater. The mean amount of zinc present in yeast from fermentations of turbid wort, or fermentations supplemented with linoleic acid and β-sitosterol or trub (original gravities 1.080 and 1.110 , respectively) did not differ significantly ($p = 0.05$) from that found in the control (Table 31). The average zinc content in yeast from fermentations supplemented with ashed trub or the soluble fraction of ashed trub was significantly greater ($p = 0.05$) than that of the control. Yeast from fermentations supplemented with EDTA-treated lipid-extracted trub displayed zinc concentrations significantly lower ($p = 0.05$) than that in the control yeast. The average magnesium content in yeast from fermentations supplemented with EDTA-treated trub, linoleic acid and β-sitosterol or trub was significantly lower than that found in the control yeast. Yeast from fermentations of turbid wort also contained significantly lower amounts of magnesium than that found in the control yeast. Fermentations supplemented with ashed trub and the soluble fraction of ashed trub increased the magnesium content of the yeast significantly ($p = 0.05$).

TABLE 30. Magnesium and zinc composition of yeasts harvested from fermentations of glucose-salts medium (original gravity 1.060) showing the effect of fermentation treatments. Values are the average of three independent determinations ± standard deviation.

Fermentation supplement	Amount (mg) magnesium (g yeast)$^{-1}$	Amount (μg) zinc (g yeast)$^{-1}$
Control	0.50 ± 0.02	195 ± 8
Ashed trub (1 g)	2.20 ± 0.05	202 ± 7
Linoleic acid and β-sitosterol[a]	0.52 ± 0.02	239 ± 15
Trub (1 g)	0.54 ± 0.02	192 ± 9
Zinc-ions[b]	0.52 ± 0.03	317 ± 13

[a] 5.4 mg linoleic acid and 1 mg β-sitosterol (200 ml medium)$^{-1}$

[b] 128 μg $ZnSO_4$ (200 ml medium)$^{-1}$

TABLE 31. Magnesium and zinc composition of yeasts harvested from fermentations of brewer's wort showing the effect of fermentation treatments. Values are the average of three independent determinations ± standard deviation.

Fermentation supplement and original gravity	Amount (mg) magnesium (g yeast)$^{-1}$	Amount (μg) zinc (g yeast)$^{-1}$
Control (O. G. 1.080)	1.05 ± 0.02	202 ± 8
Ashed trub (1 g; O. G. 1.080)	1.71 ± 0.03	284 ± 12.7
EDTA-treated lipid-extracted trub (1 g; O. G 1.080)	0.50 ± 0.03	133 ± 7
Linoleic acid and β-sitosterol[a](O. G. 1.080)	0.88 ± 0.01	228 ± 10
Soluble fraction of ashed trub (O. G. 1.080)	1.52 ± 0.03	256 ± 16
Trub (1 g; O. G. 1.080)	0.51 ± 0.02	191 ± 12
Turbid wort (O. G. 1.080)	0.54 ± 0.02	201 ± 7.1
Control (O. G. 1.100)	1.06 ± 0.04	231 ± 18
Trub (1 g; O. G. 1.100)	0.51 ± 0.02	209 ± 20

[a] 5.4 mg linoleic acid and 1 mg β-sitosterol (200 ml medium)$^{-1}$

ZINC COMPOSITION OF BREWER'S WORT AND GLUCOSE-SALTS MEDIUM

Glucose-salts medium (original gravity 1.060) contained 284 ± 17 μg zinc (l medium)$^{-1}$. Brewer's wort (original gravity 1.080) contained 376 ± 60 μg zinc (l medium)$^{-1}$.

MAGNESIUM AND ZINC COMPOSITION OF STARTER YEAST

Yeast was harvested from starter cultures of glucose-salts medium (original gravity 1.060), freeze-dried and analysed for magnesium and zinc as already described in the Methods Section. Starter yeast contained 1.33 ± 0.04 mg magnesium (g yeast)$^{-1}$ and 156 ± 18.4 μg zinc (g yeast)$^{-1}$.

END-POINT ETHANOL DETERMINATIONS FOR FERMENTATIONS OF BREWER'S WORT AND GLUCOSE-SALTS MEDIUM BY *SACCHAROMYCES CEREVISIAE*

The ethanol yield, from fermentations of glucose-salts medium (original gravity 1.060) supplemented with trub, was significantly greater ($p = 0.05$) than that from all other fermentations of that medium except for those supplemented with the water insoluble extract of trub (Table 32). The ethanol yield from fermentations of glucose-salts medium (original gravity 1.080) supplemented with trub was significantly greater ($p = 0.05$) than that of the control. All treatments to fermentations of glucose-salts (original gravity 1.060), bar those supplemented with ashed trub and zinc displayed a significantly greater($p = 0.05$) ethanol yield than the control. Fermentations of brewer's wort (original gravity 1.060) by yeast supplemented with trub did not produce significantly greater ($p = 0.05$)

TABLE 32. End-point ethanol determinations for fermentations of glucose-salts media (original gravity 1.060 unless stated) showing the effect of fermentation treatments. Values are the average of three independent determinations ± standard deviation.

Fermentation supplement	Ethanol concentration percentage by volume
Control	2.30 ± 0.18
Trub (1 g)	7.58 ± 0.15
Fresh trub (1 g)	4.53 ± 0.53
Fresh trub (2 g)	5.23 ± 0.22
Control (O. G. 1.080)	2.68 ± 0.38
Trub (1 g; O. G 1.080)	8.52 ± 0.36
Ashed trub (1 g)	2.56 ± 0.30
Lipid-extracted trub (1 g)	6.27 ± 0.31
EDTA-Treated trub (1 g)	3.51 ± 0.35
EDTA-Treated lipid-extracted trub (1 g)	3.01 ± 0.20
Lipid extract of trub (1 ml)	3.34 ± 0.42
Water soluble trub	4.83 ± 0.45
Water insoluble trub (1 g)	7.34 ± 0.60
Linoleic acid and β-sitosterol[a]	3.35 ± 0.48
Zinc-ions[b]	2.52 ± 0.46

[a] 5.4 mg linoleic acid and 1 mg β-sitosterol (200 ml medium)$^{-1}$

[b] 128 μg $ZnSO_4$ (200 ml medium)$^{-1}$

ethanol yields than control fermentations (Table33). At higher specific gravities, 1.080 and 1.100 , respectively, fermentations of brewer's wort with trub supplemented produced significantly greater yields of ethanol than control fermentations. However, when ethanol yields from control fermentations of brewer's wort (original gravity 1.080) were compared with those obtained from fermentations with supplements other than trub no significant differences ($p = 0.05$) were apparent. An exception was the ethanol yield of soluble ash supplemented fermentations which was significantly lower ($p = 0.05$) than that of the control.

ETHANOL TOLERANCE OF YEAST FROM BREWER'S WORT AND GLUCOSE-SALTS MEDIUM FERMENTATIONS

Fermentation treatment had no effect on the ethanol tolerance of the yeast under the conditions used. Yeast from stuck fermentations was able to grow at the same concentration of ethanol as yeast from supplemented fermentations (Table 34).

TABLE 33. End-point ethanol determinations for fermentations of brewer's wort (original gravity 1.080 unless stated) showing the effect of supplemented. Values are the average of three independent determinations ± standard deviation

Fermentation supplement	Ethanol concentration percentage by volume
Control (O. G. 1.060)	6.03 ± 0.12
Trub (100 mg; O. G. 1.060)	6.01 ± 0.19
Trub (250 mg; O. G. 1.060)	6.05 ± 0.24
Trub (1 g; O. G. 1.060°)	6.11 ± 0.13
Control	7.81 ± 0.16
Trub (1 g)	8.50 ± 0.21
Control (O. G. 1.1000)	7.45 ± 0.28
Trub (1 g; O. G. 1.100)	8.42 ± 0.31
Turbid wort	7.51 ± 0.34
Lipid extracted trub (1 g; O. G. 1.060)	6.05 ± 0.12
Ashed trub (1 g)	8.03 ± 0.51
Soluble fraction of ashed trub (10 ml)	7.31 ± 0.14
EDTA-Treated lipid-extracted trub (1 g)	7.61 ± 0.32
Linoleic acid and β-sitosterol[a]	7.73 ± 0.12
Activated charcoal (1 g)	7.43 ± 0.21

[a] 5.4 mg linoleic acid and 1 mg β-sitosterol $(200 \text{ ml medium})^{-1}$

TABLE 34. Ethanol tolerance of yeasts from brewer's wort (original gravity 1.080 unless otherwise stated) and glucose-salts medium (original gravity 1.060) fermentations. Tolerance was measured as the maximum ethanol concentration that allowed growth in a 1.060 specific gravity glucose-salts medium.

Fermentation supplement and yeast origin	Maximum concentration of ethanol that allowed growth. Percentage by volume
Control (glucose-salts)	6
Trub (1 g; glucose-salts)	6
Ashed trub (1 g; glucose-salts)	6
Linoleic acid and β-sitosterol[a] (glucose-salts)	6
Zinc-ions[b] (glucose-salts)	6
Control (wort)	6
Trub (1 g ; wort)	6
Control (1 g; wort; O. G 1.100)	6
Trub (1 g; wort; O. G 1.100)	6
EDTA-Treated lipid-extracted trub (1 g; wort)	6
Turbid wort (wort)	6

[a] 5.4 mg linoleic acid and 1 mg β-sitosterol (200 ml medium)$^{-1}$

[b] 128 μg $ZnSO_4$ (200 ml medium)$^{-1}$

DISCUSSION

Essentially, brewing is a high-volume low-profit industry and, as such, brewer's have striven for greater economy in the production of their beers. In the modern brewery, the repitching of washed crop yeasts, thus negating the need for expensive fresh propagation, the utilisation of spent grains for animal feeds, and the use of higher adjunct ratios in the mash, are just some of the means of cost-saving. However, over the last 20 years much research has centred on high-gravity brewing which, amongst other benefits, enables the use of existing plant to make potentially enormous increases in brewing capacity, by brewing a stronger than normal base beer and diluting it to the required strength. However, problems have arisen through the use of worts with a high specific gravity, and are associated with over-production of esters (Whitworth, 1978) and stuck fermentations (Day *et al.*, 1975). Sluggish or stuck fermentations have been attributed to a number of factors, namely, ethanol toxicity (Day *et al.*, 1975), high osmotic pressure (Panchal *et al.*, 1982), lack of nutrients (Casey *et al.*, 1983), carbon dioxide inhibition (Siebert *et al.*, 1986) and a build up of toxic by-products (Lafon-Lafourcade *et al.*, 1984).

TRUB COMPOSITION

Literature reports suggest that lipid, protein and tannin are major components of trub (Hough *et al.*, 1982; Vernon, 1984). The results presented in this thesis are in broad agreement with the literature observations, but also show that the inorganic component of the hot-break trub studied to be much greater than that previously reported. Carbohydrate is shown to be a minor component of trub.

Of particular interest in this study are the components of trub which can contribute to improved yeast fermentation performance, namely metal ions, sterols and unsaturated fatty acids. This study has shown, along with others, that there are considerable quantities of such components in trub (Anness and Read, 1985 a, b;

Jones *et al.*, 1975; Schisler *et al.*, 1982). Generally, hot-break trub contained a greater amount of lipid than cold-break trub, which reflects the findings that large amounts of wort lipids are lost during wort boiling (Anness and Read, 1985 b). Although trub was collected from brews of the same grist in this study, there was considerable variation in the fatty-acyl composition between replicates. This reflects the heterogeneous nature of trub.

FERMENTATION OF GLUCOSE-SALTS MEDIUM AND BREWER'S WORT

In the experiments performed in this study, trub was found to stimulate the fermentation of both brewer's wort and glucose-salts medium. Stimulation was characterised by more rapid and greater attenuation, greater ethanol production,greater suspended yeast counts and growth in fermentations to which trub was added. It was apparent, at high specific gravity, that trub was required for complete attenuation of glucose-salts medium and for rapid attenuation of brewer's wort. At specific gravities of 1.060 and 1.080, respectively, trub was a prerequisite for attenuation of glucose-salts medium. The absence of trub from fermentations of brewer's wort resulted in sluggish fermentations. Any attempt at explaining the stimulative effect of trub in fermentation needs to consider the reasons why a fermentation should become stuck. What is important is that whatever the reason for stuck fermentations, the presence of trub overcomes the problem.

Glucose-salts medium

In the case of glucose-salts medium of the possible explanations for stuck fermentations outlined earlier, ethanol toxicity can be ruled out since, at the point of the fermentation sticking, the concentration of produced ethanol was low. The argument in favour of high osmotic pressure contributing to failure of the yeast to attenuate the medium, through intracellular accumulation of ethanol, would appear tenuous. It is apparent that high substrate concentrations do not lead to intracellular

ethanol accumulation, since transport of ethanol into and out of yeast cells is not affected by high osmotic pressures (Guijarro and Lagunas, 1984). Despite this, some evidence from fermentations of maltose-salts medium seems to support high osmotic pressure as a cause for stuck fermentations. An explanation for this could be that maltose, a disaccharide, exerts a lower osmotic pressure per mole than glucose. Since the yeast used was a brewer's yeast, it may intrinsically utilise maltose at a faster rate than glucose.

Glucose-salts media of specific gravity 1.060 and 1.080, respectively, were prepared by increasing the concentration of glucose in the medium, the concentrations of other essential nutrients, such as amino-nitrogen, dissolved oxygen, metal ions, unsaturated fatty acids and sterols remained unaltered (the concentration of dissolved oxygen was lower due to the increase in osmotic pressure). Therefore, the greater amount of fermentative work, and hence growth, required from the yeast to ferment such media assumes an excess of the other essential nutrients. However, any one of these essential nutrients may become limiting, therefore accounting for stuck fermentations. The presence of trub may overcome the deficiency and allow yeast growth to occur. A similar nutrient-limitation theory has been proposed to account for the failure of brewing yeast to attenuate high-gravity worts prepared by supplementing malt-wort with high-sugar syrups (Casey *et al.*, 1983).

The stimulative effect of trub has been attributed to its physical presence acting as a site for carbon dioxide evolution (Siebert *et al.*, 1986). Carbon dioxide supersaturation has been shown to occur in fermenting worts (Trolle, 1950; Delente and Gurley, 1968). During fermentation, as yeast falls out of suspension, carbon dioxide supersaturation may occur in the sediment long before saturation is reached in the main liquid body. Nucleation of carbon dioxide bubbles occurs at the bottom of the fermenter, at nucleation sites provided by wort solids in fermenting beer (Delente *et al.*, 1969). Since the glucose-salts medium was free from solids, except where trub was added, the non-availability of nucleation sites for carbon dioxide evolution, resulting in localised carbon dioxide supersaturation, may account for the problems

encountered with fermentations at high specific gravity. However, it is arguable wether the dissolved carbon dioxide concentration, at the point when fermentations stuck, is sufficient to arrest fermentation.

Studies suggested that the observed inhibition of fermentations was, to a large degree, reversible, since the addition of trub to stuck fermentations enabled the fermentation to restart. Similar effects have been recorded in fermentations of wine musts, where stuck fermentations were restarted by adding yeast hulls to the must (Lafon-Lafourcade *et al.*, 1984; Ribereau-Gayon, 1985; Munoz and Ingledew, 1989 a, b). It is claimed in these reports that yeast hulls work in two ways. Firstly they absorb potentially lethal medium- and short-chain fatty acids produced by the yeast during fermentation; secondly they act as a source of lipids which may be used to prolong cell growth and viability. It is feasible that trub has a similar role in fermentations of glucose-salts medium. However, control fermentations often stuck after a short period of fermentation, and it is arguable whether toxic by-products produced by the yeast are present in sufficient concentrations to arrest growth at such an early stage.

An important point to take into account when considering the reasons for stuck fermentations of glucose-salts medium at specific gravity 1.060 is the variablity in the response of the control fermentations. Given that the medium was the same in each case the only nutrient that could fluctuate in concentration was oxygen. Although media was prepared and sterilised as constantly as possible to keep variation to a minimum it would appear that fluctuations in the dissolved oxygen concentration occurred.

Recently, ionic deficiencies in laboratory media have been cited as reasons for poor yeast fermentation performance (Dombek and Ingram, 1986 b; Nabias *et al.*, 1988). Trub may serve to correct any such deficiency in the glucose-salts medium used.

When one reflects on these theories regarding stuck fermentations and the problems encountered in the experiments discussed here, one is drawn to the likelihood that trub plays a nutritional role in stimulating fermentation of brewer's

wort and glucose-salts medium. It has long been known that, under anaerobic conditions, yeast requires an exogenous supply of sterol and unsaturated fatty acid for growth to occur (Andreasen and Stier, 1953, 1954). Wort fatty acids are known to be absorbed by yeasts during fermentation (Suomolainen and Keranen, 1968). Long-chain unsaturated fatty acids either in wort, or supplemented during fermentation, have been shown to have a beneficial effect on yeast performance (Hayashida *et al.*, 1974, 1975; Thomas *et al.*, 1978; Thomas and Rose, 1979;Casey *et al.*, 1983, 1984; Ingledew and Kunkee, 1985). These reports assume greater significance when one considers the essentially anaerobic nature of the fermentations reported in this study. It is of interest to appreciate the oxygen credit for glucose-salts medium fermentations. This is made up of three components, namely the sterol and unsaturated fatty-acyl reserves of the pitching yeast, the dissolved oxygen present in the medium, and finally those preformed components in the medium that require oxygen for their synthesis. The last component was shown to be negligible in the case of control fermentations, but would include trub lipids in trub-containing fermentations. Therefore, in control fermentations, the oxygen credit is made up of only the unsaturated fatty-acyl and sterol reserves of the pitching yeast and dissolved oxygen present in the medium. Results confirmed that the pitching yeast had a large pool of sterol and mono-unsaturated fatty-acyl residues ready for utilisation during anaerobic growth. No $C_{18:2}$ residues or β-sitosterol were found, confirming that the yeast used in these studies was unable to synthesize such lipids. Given that oxygen solubility decreases as specific gravity increases, it is possible, at a specific gravity of 1.060 and above, that the oxygen credit is not sufficient to allow enough yeast growth to enable attenuation of the medium. Yeast in fermentations to which trub had been added was shown to draw upon the preformed unsaturated fatty acids and sterols therein to redress the oxygen credit, allowing yeast growth and attenuation of the medium. Such results are in accordance with those of Schisler and co-workers (1982) who demonstrated that worts with high contents of trub (2.66% w/v) could dispense with, in part, aeration regimes and give comparable fermentation performance. It was

apparent that trub was responsible for the differences in the Δ mol^{-1} values and types of fatty-acyl residues and sterols found in yeast cells from control and trub-containing fermentations. It can be confidently assumed that trub lipids, particularly $C_{18:2}$ and β-sitosterol, having been incorporated into the yeast, have a beneficial effect on fermentation. In this respect, the evidence indicates that trub has a nutritive effect in stimulating fermentation of glucose-salts medium. Previously, workers have shown that wort sterols and unsaturated fatty acids were incorporated into yeast cells (David and Kirsop, 1972, 1973). This report clearly shows that trub sterols and unsaturated fatty acids are taken up by yeast cells during fermentation.

It was clear that the dissolved oxygen concentration in glucose-salts medium was low because of the large amounts of the sterol precursor squalene in yeasts from all fermentations. Such accumulation of squalene has long been known to occur in yeast grown under anaerobic conditions (Jollow *et al.*, 1968; Klein, 1955).

Further less direct evidence of the involvement of trub lipids in its stimulative action comes from fermentations supplemented with the water-insoluble extract of trub. Such an extract, which one assumes contains the vast majority of the lipid components of the trub and any tightly bound ions, was able to stimulate fermentation to almost the same degree as trub. The water-soluble fraction of trub did not stimulate fermentation to the same degree as trub, suggesting that those components readily soluble in water, although possessing a capacity to stimulate yeast fermentation, are not solely responsible for the improvements in performance associated with the presence of trub. It appears, therefore, that the components responsible for trub's stimulative action are not readily dissolved in water, implying that they are organic and probably lipidic in nature.

Given such compelling evidence suggesting a nutritive role for trub lipids in stimulating glucose-salts medium fermentations, the inability of either the lipid fraction of trub or lipid supplements to promote full attenuation was puzzling. It was clear that sterols and unsaturated fatty acids from the trub lipid extract and the lipid supplements were taken up by yeast. It was interesting to note that lipid-extracted trub

stimulated greater attenuation of glucose-salts medium than either the trub lipid extract or lipid supplements. However, as expected, yeast from such fermentations did not contain polyunsaturated fatty-acyl residues or β-sitosterol. These results support the report of Siebert *et al.* (1986) who demonstrated that the lipid extract of trub was less effective than the non-lipid residue at stimulating fermentations of wort. Similarly, Viegas *et al.* (1985) showed that the aqueous fraction of soy flour was more efficient at stimulating fermentation of glucose-salts medium than the lipid fraction. The failure of the lipid supplements, linoleic acid and β-sitosterol, to stimulate complete attenuation would suggest that it is not, solely, through lack of sterol and an unsaturated fatty acid, and hence by implication oxygen, that control fermentations of glucose-salts medium failed to attenuate.

The role of the ionic components of trub in stimulating fermentation appears to be debatable. A deficiency in the concentration of zinc ions, an important trace requirement essential for yeast growth, was suspected. However, both glucose-salts medium and starter yeast contained sufficient concentrations of zinc ions (Jones and Greenfield, 1984a) to suggest that zinc-related growth problems would not be encountered.

The effect of a strong chelator, such as EDTA, on trub would be to remove ions from it, even those chelated to the various organic compounds present. Therefore, in effect the treatment leaves the organic fraction intact while removing much of the inorganic fraction. When one considers this, it can be deduced that the ionic component of trub is an important factor in stimulation of fermentation. However, the ashed fraction of trub, from which all of the organic material had been removed, did not stimulate fermentation to a great extent. Given this it can be argued that the stimulative action of trub displayed in these fermentations was not due to the correction of a simple ionic deficiency. Since the magnesium and zinc contents of yeast from control and trub-supplemented fermentations of glucose-salts medium were not significantly different, it was inferred that a deficiency in either of these ions is not responsible for the occurrence of stuck fermentations. Although significantly

greater intracellular zinc-ion concentrations were found in yeasts from lipid-supplemented and zinc-ion supplemented fermentations, they had no beneficial effect on fermentation. Likewise, a significantly greater concentration of magnesium ions in ashed trub-supplemented fermentations did not stimulate fermentation. The intracellular zinc concentrations reported here are greater than those of Lentini *et al.* (1990), although these differences may be attributed to the types of media used and the zinc-ion requirements of the respective yeast strains. Supplementation of fermentations with copper ions, manganese ions and zinc ions did not effect attenuation of the medium. The results presented here would rule out the theory, as advocated by Dombek and Ingram (1986 b), that complex nutrients merely corrected ionic deficiencies in the fermentation medium.

The argument for a purely physical effect to explain the stimulative action of trub would appear to be unfounded since EDTA-treated lipid-extracted trub did not stimulate fermentation to a great extent, and activated charcoal hardly at all. Nevertheless, the stimulative effect of EDTA-treated lipid-extracted trub was enough to suggest that the provision of nucleation sites for carbon dioxide evolution may play a part in the stimulative action of trub. An important point to consider is the possible effect that the extraction procedures (chloroform-methanol and EDTA-treatment) have upon the physical nature of the trub. Given the essentially lipid and proteinaecous nature of trub, extraction of components soluble in organic solvents, or of metal ions, could have profound effects on the conformation and structure of the trub residue.

A physical effect due to the absorption of medium- and short-chain length fatty acids was not indicated by these results, since fermentations to which extracted trub solids were added did not ferment to the same degree. It is possible that extraction procedures altered the absorptive capacity of the trub. However, given the anaerobic nature of the fermentation, the nutritional deficiencies of the medium (for sterols and unsaturated fatty acids) and the results discussed above, it is difficult to

see how absorption of toxic medium- and short-chain fatty acids could account for the stimulative action of trub.

Evidence implicating a physical role for trub in stimulation comes from the greater suspended yeast counts in fermentations to which trub or trub solids were added. Such an effect could be attributed to a greater amount of fermentative activity in such fermentations. However, the stirring effect in a static fermentation is brought about by the evolution of carbon dioxide once the medium is saturated with the gas. Gas evolution requires a rough surface with cavities which allow bubble growth to occur. The supplementation of fermentations with solids would provide such a rough surface. It would appear that the sucess of a supplement lies in both its nutritive potenetial, to stimulate growth, and its physical potential, to nucleate carbon dioxide bubbles and so create a stirring motion. The evolution of gas bubbles helps keep yeast, and trub, in suspension where the nutritive benefits of trub can be exploited. Furthermore, the presence of solid particles such as trub may concentrate nutrients at the liquid-solid interface as suggested by Merritt (1967). The solid in question, namely trub, has a considerable reserve of lipid material of potential benefit to yeast growth.

Further circumstantial evidence for a physical effect to explain trub's stimulative action comes from the relative fermentation performances of yeast from fermentations supplemented with ashed and lipid-extracted trub. As expected yeasts from fermentations supplemented with ashed and lipid-extracted trub did not contain quantifiable amounts of $C_{18:2}$, or $C_{18:3}$ fatty-acyl residues, or β-sitosterol since neither supplement contained a large lipid component. Indeed, in the case of ashed trub the entire organic component of trub had been eliminated. The trace amounts of such lipids could be attributed to contamination of the sample, integrator error, or residual lipid in the lipid-extracted trub solids. It was noticeable that the control response was better in the ashed trub-supplemented fermentation than in the lipid-extracted trub-supplemented fermentations. Taking into account the earlier argument, that only the dissolved oxygen content in fermentations could vary, this would

suggest that the dissolved oxygen content was slightly greater in fermentations supplemented with ashed trub than in fermentations supplemented with lipid-extracted trub. Given that the Δ mol^{-1} values were similar in yeast supplemented with ashed trub (0.43) and lipid-extracted trub (0.45), the difference in fermentative performance could not be due to the presence of lipid nutrients, or differences in dissolved oxygen concentration and, since both supplements retain the ionic fraction of trub, the failure of ashed trub-supplemented fermentations to attenuate would seem to be related to the lack of sites for carbon dioxide evolution. This suggests that the physical presence of the lipid-extracted trub had some bearing on the comparative improvement in fermentation.

To obtain a greater understanding of what effects the respective trub fractions have upon fermentation, it is necessary to know their physical nature and nutritive potential, and to determine their fermentation performance against that of trub-supplemented fermentations. An interesting finding is that the ionic and lipid components of trub are more effective at stimulating fermentations when solids are present than they are on their own. For example, EDTA-treated trub, which contains the lipid fraction in the residual solids, is more efficient at stimulating fermentation than the lipid extract of trub. Similarly, lipid-extracted trub, which would retain the ionic component of trub in the residual solids, was more effective than the water-soluble extract at stimulating fermentation. This provides evidence for an interaction between the physical and nutritive attributes of trub in its stimulative action.

Since no one crude extract from trub elicited the same effect as trub itself, these studies provide further evidence to suggest that an explanation of the stimulative action of trub on fermentation involves both nutritive and physical effects.

It is likely that the improved fermentation performance of yeast repitched from trub-containing fermentations, as opposed to yeast repitched from control fermentations, was due to the former absorbing nutrients from the trub. Only a nutritive effect could be carried over from a previous fermentation. This suggests such a nutritional effect to be important in trub's stimulation of fermentation. An

interesting point to note was the better fermentation performance of yeast repitched from stuck fermentations into fresh medium. It may be inferred that the yeast had adapted to its environment, but it is more likely that harvesting, washing and refrigerating the yeast at 4°C had a beneficial effect on yeast metabolism. During these operations, exposure to air could enable the yeast to absorb oxygen and use it to synthesize sterols and unsaturated fatty acids. This may account for the improved fermentation performance of yeast repitched from control fermentations.

Since supplementation of fermentations with trub resulted in the greatest degree of attenuation, and provided that trub did not stimulate production of yeast mass rather than ethanol, it is not surprising that the greatest ethanol concentrations occurred in fermentations containing trub. As expected, where supplements enabled the yeast to attenuate a greater proportion of the medium than the control, the ethanol yield was greater. These results suggest that trub stimulates production of ethanol.

It was apparent that fermentation treatment had no effect on the ethanol tolerance of the yeast. However there are a number of problems with the experimental design. These include, the small quantities used, the difficulty in covering the microtitre plates to prevent evaporation of ethanol and the failure to take into account the ethanol produced over the duration of the growth period. Even given these drawbacks, it was remarkable to record in these experiments that the previous history of the yeast had no bearing on its ability to withstand an ethanol challenge.

Brewer's wort

The findings presented here confirm the reports of Schisler *et al.* (1982) and Siebert *et al.* (1986) who showed that trub stimulates the fermentation of wort, by stimulating greater growth, greater suspended yeast counts and greater ethanol yields than in unsupplemented fermentations.The effect of trub on the fermentation of brewer's wort is less dramatic than its effect on glucose-salts medium, which is not surprising given that brewer's wort, with the exception of some amino-nitrogen acids, generally contains an excess of all components necessary for growth (Brown and

Kirsop, 1972). In fermentations involving glucose-salts medium, oxygen deficiency was suspected as being a limiting factor. Preformed unsaturated fatty acids and sterols present in the wort contribute significantly to the oxygen credit in such fermentations. Therefore, the requirement for dissolved oxygen is decreased in comparison to that in glucose-salts fermentations. This would account for the rare occurrence of completely stuck fermentations.

Since the amount of trub supplemented to a fermentation affected the degree of stimulation, the argument that trub has a nutritive role in stimulating fermentation of wort is persuasive. Indeed, Schisler *et al.*, (1982) proposed that the stimulative action of trub in wort fermentations was due to the combined nutritive effect of unsaturated fatty acids, sterols and zinc-ions. Evidence from the work presented here seems to support such an hypothesis, at least in the case of the lipid nutrients. Yeast from trub-supplemented fermentations contained twice as much $C_{18:2}$ as yeast from control fermentations, and given the evidence available from fermentations of glucose-salts medium, it would appear that $C_{18:2}$ residues are incorporated into yeast cells from the trub as well as from the wort itself. It is known that linoleic acid from wort (David and Kirsop, 1972, 1973) is incorporated into yeast. β-Sitosterol is taken up by yeast from worts supplemented with spent grain lipids (Taylor *et al.*, 1979). These reports suggest that linoleic acid and β-sitosterol are taken up by yeast from the trub as well as the wort. There was no evidence to suggest that zinc-ions from trub stimulated fermentation since the concentrations of this ion in control and trub-supplemented yeast were not significantly different. It was shown that the zinc concentration in the wort was sufficient, above 0.2 p.p.m. (Jones and Greenfield, 1984), to suggest that zinc-related growth problems would not occur. The stimulative influence of trub in fermentations of wort does not apppear to be through the provision of magnesium ions.

Supplementation of fermentations with linoleic acid and β-sitosterol stimulated the initial phase of fermentation. This concurs with the reports of Casey *et al.* (1983, 1984), who demonstrated the nutritive effect of ergosterol, oleic acid and

yeast extract on the fermentation of a high specific gravity wort (1.133). However trub-supplemented fermentations attenuated to a greater degree than lipid supplemented fermentations. This is somewhat surprising since the lipids supplemented, namely linoleic acid and β-sitosterol, are known to be incorporated from trub into yeast and are thus implicated in improving fermentation performance. Indeed yeast supplemented with linoleic acid and β-sitosterol were shown to take up more of those lipids than trub-supplemented yeast. These results suggest that the stimulative action of trub is more complex than a nutritive hypothesis based purely on the ability of trub lipids to substitute for oxygen. Similar findings were reported by Siebert *et al.* (1986), who found that supplementation of wort with the lipid extract of trub, while stimulating fermentation, did not stimulate to the same degree as trub. These workers also found that addition of ergosterol and linoleic acid to worts had little or no effect on fermentation, whereas addition of insoluble lecithin had the greatest stimulative effect. Siebert *et al.* (1986) also demonstrated increased fermentation efficiency, greater yeast viability and cell numbers in fermentations of wort supplemented with activated carbon as compared to those supplemented with trub solids. However, similar experiments during this study showed no such advantage in fermentation performance, rather the opposite.

It is likely that the concentration and composition of the trub found in turbid wort was not sufficient to stimulate fermentation. The trub found in turbid wort is composed of some hot-break trub, carried over from the whirlpool, and the cold-break that comes out of solution during wort cooling. Lipid analysis showed cold-break trub to have considerably less $C_{18:2}$ fatty-acyl residues and β-sitosterol associated with it than hot-break trub. Although the amount of trub present in turbid worts was not measured, it was considerably less than that present in fermentations of clarified wort containing hot-break trub.

An interesting finding was that the Δ mol^{-1} value for yeast from fermentations supplemented with EDTA-treated lipid-extracted trub (0.64), was greater than the corresponding value for yeasts from fermentations supplemented with other trub

fractions. The contribution of lipids from EDTA-treated lipid-extracted trub to the lipid pool in the wort can be regarded as negligible. Therefore, the presence of the solid trub residue would appear to stimulate uptake. It is likely that the improved fermentation performance of yeast from EDTA-treated lipid-extracted fermentations, as compared with yeast supplemented with other trub fractions, is due to a greater concentration of unsaturated fatty-acyl residues present in the yeast. These unsaturated fatty-acyl residues are probably incorporated into the plasma membrane and contribute to the maintenance of optimal membrane fluidity, thereby improving the fermentation performance of the yeast. An interesting result was the increased proportion of $C_{18:1}$ fatty-acyl residues detected in yeast from fermentations supplemented with the soluble fraction of ashed trub. This is unusual since, although $C_{18:1}$ residues are present in wort and trub, it is the second most abundant unsaturated fatty-acyl residue behind $C_{18:2}$ (Jones *et al.*, 1975; Anness and Reed, 1985 a, b). This may reflect stimulation of unsaturated fatty-acyl synthesis by magnesium ions (Jones and Greenfield, 1984) promoting more efficient utilisation of the traces of oxygen in the wort, and since *Saccharomyces cerevisiae* have only a Δ^9 desaturase (Ratledge and Evans, 1987), only $C_{18:1}$ can be produced.

There is some circumstantial evidence which appears to support the theory that trub acts to nucleate carbon dioxide. Both lipid-extracted trub and ashed trub stimulated fermentation compared to control fermentations, suggesting a role for both the ionic and lipidic components of trub in eliciting this effect. However, the comparatively minor stimulative effect of the soluble fraction from ashed trub on wort fermentation, implies that the positive effect induced by the ashed trub was not an ionic effect but a physical one, attributed to the insoluble ash providing nucleation sites for carbon dioxide evolution. Further evidence alluding a physical effect to explain trub's stimulative action comes from fermentations supplemented with EDTA-treated lipid-extracted trub, in which the nutritive components of trub were virtually removed, which proved almost as stimulative as trub. Indeed, a recent report suggests that trub stimulates fermentation by providing nucleation sites for carbon

dioxide evolution, therefore alleviating carbon dioxide supersaturation of the wort and the attendant inhibition of yeast cells (Siebert *et al.*, 1986).

The nature of the stimulative effect of trub in wort fermentations would appear to be nutritive, through the provision of sterols and unsaturated fatty acids, which complement the pool of such lipids in the wort, thus offsetting the decreased solubility of oxygen in high-gravity worts. There are some results that hint at the possibility of a physical effect. Generally, though not in all cases, suspended yeast counts were greater in fermentations to which trub or extracted trub solids had been added. This suggests a physical effect most likely to be through trub providing a rough surface upon which nucleation of carbon dioxide occurs once the wort is saturated with carbon dioxide$_{(\text{aqueous phase})}$. As the gas is released it may carry yeast cells and / or trub, thus creating a stirring motion in the fermenter. This stirring action would help to keep yeast cells in suspension.

CONCLUSIONS

The findings presented here confirm that trub stimulates fermentations of brewer's wort and glucose-salts medium. Fermentations which had been supplemented with trub attenuated to a greater degree, fermented more rapidly, had a greater number of yeasts in suspension and, had greater growth and ethanol yields than those that were not supplemented. There is some disagreement in the literature as to the nature of the stimulative effect of trub in fermentations. Schisler *et al.* (1982) proposed a nutritional effect, whereas Merritt (1967) and Siebert *et al.* (1986) attribute the stimulative effect of trub to physical effects. The evidence from the work presented here suggests that the stimulative action of trub is due to its nutritive content. Yet there are hints that the physical presence of trub has a beneficial effect on fermentation.

It is likely that oxygen is the limiting factor in fermentations of and glucose-salts medium. At specific gravities of 1.04 and 1.05 there is sufficient oxygen present

for complete fermentation of the medium. The presence of trub accelerates fermentation by providing extra sterol and unsaturated fatty acid supplements. As the specific gravity of the medium increases so the solubility of oxygen decreases. At a specific gravity of 1.06 there would not appear to be sufficent oxygen credit in the medium and pitching yeast to allow complete attenuation of the medium. It was shown that trub provided the necessary nutrients, a sterol and unsaturated fatty acids, to enable complete fermentation. However, fermentations which were supplemented with the trub lipid fraction or lipid supplements did not stimulate complete attenuation of the medium, even though the lipids were taken up by the yeast. This coupled with evidence from suspended yeast counts, which were greater in trub-supplemented fermentations, implies a physical effect is important in trub's stimulative action. Similar experiments using brewer's wort appeared to suggest that trub lipids, though important in determining trub's stimulative action, were not wholly responsible.

It is clear that during fermentation sterol and unsaturated fatty acids, particularly β-sitosterol and linoleic acid, are taken up by yeast and, presumably incorporated into the yeast plasma membrane. In this respect trub is acting as a source of nutrients, replacing the need for a preformed streol and unsaturated fatty acid or oxygen in the medium. However, it is evident that such supplementation alone does not account for the stimulative action of trub. The greater suspended yeast counts in fermentations in the presence of trub, or extracted trub solids, suggests a physical effect, probably related to the evolution of carbon dioxide. The indications are that a supplement needs to satisfy both requirements, the nutritional, for a sterol and an unsaturated fatty acid, and the physical, for a rough surface for carbon dioxide evolution, for it to stimulate fermentation.

There are a number of experiments that would complement and extend the present study. Among these would be regulation of the dissolved oxygen content of the media uesd observing the effects of trub supplementation on fermentation. The effect of varying the dissolved oxygen content could also be investigated. Further studies are required to confirm the implied physical effects of trub in stimulation of

fermentation. Since nucleation of carbon dioxide appears to be a factor, measurement of the dissolved carbon dioxide concentration may reveal additional information regarding the effiacy of supplements at nucleating carbon dioxide and shed further light on the proposal of Siebert *et al.* (1986), who suggested that trub acts to alleviate carbon dioxide-induced growth inhibition. An extra area for study be would involve analysis of esters and fusel alcohols since it is apparent that flavour trials favour beers produced from clarified rather than high-trub worts (Schisler *et al.*, 1982). However, investigations may reveal an optimal concentration for trub in wort that will enable stimulation of fermentation and not affect the organoleptic qualities of the product. This study has demonstrated that trub has an important role to play in fermentations of high-gravity worts and has highlighted the significance of particulate material in fermentations in general.

PART TWO: UPTAKE OF FATTY ACIDS BY *SACCHAROMYCES CEREVISIAE*

INTRODUCTION

Under anaerobic conditions yeast cell growth is dependent upon the supply of an exogenous unsaturated fatty acid, either palmitoleic, oleic, linoleic or linolenic acid, in addition to a sterol and some vitamin requirements. Most studies of fatty-acid uptake by microbes have centred on *Esherichia coli* and have found that the process involves expenditure of metabolic energy to activate transport by a membrane-bound acyl CoA synthetase (Vanderwinkel *et al.*, 1968; Klein *et al.*, 1970; Frerman and Bennet 1973). Genetic evidence points to the involvement of a membrane-associated carrier protein (Nunn *et al.*, 1979).

In mammalian cells, a dual mode of fatty acid transport across cell the membrane has been proposed for for fatty acids ranging from C_6 to C_{22}. This involves both carrier mediated transport and simple diffusion (Samuel *et al.*, 1976; Chow and Hollander, 1978). Evidence suggests the existence of a similar dual mode transport system for C_{12}-C_{18} fatty acids in yeast (Kohlwein and Paltauf, 1983). Uptake of fatty acids was followed in two strains, *Saccharomyces uvarum* (*cerevisiae*), a facultative anaerobe unable to utilise fatty acids by β-oxidation, and *Saccharomycopsis lipolytica* which can utilise fatty acids as a carbon source. Kohlwein and Paltauf (1983) showed that exogenously supplied lauric and oleic acids were rapidly taken up by yeast cells with subsequent incorporation into phospholipids. The rate of oleic acid uptake was linear with *Saccharomycopsis lipolytica* up to 30 s and *Saccharomyces uvarum* (*cerevisiae*) up to 2 min. Uptake kinetics were consistent with a dual mode of transport, comprising a carrier molecule with K_T values in the range 10^{-5}-10^{-6} M, and apparent simple diffusion that predominates at high substrate levels. The rate of lauric acid uptake, using concentrations in the range 7 μM and 18 μM , was unaffected in cells treated with the uncouplers 2,4-ditrophenol or CCCP, or cells treated with the ionophore valinomycin. This indicated that uptake of fatty acids was independent of an electrochemical gradient across the plasma membrane (Kohlwein and Paltauf, 1983). It was

demonstrated that at least two fatty-acid carrier systems operated in both *Saccharomycopsis lipolytica* and *Saccharomyces uvarum* (*cerevisiae*), one for fatty acids with 12 or 14 carbon atoms, respectively, and one for C_{16} and C_{18} saturated or unsaturated fatty acids. Fatty acids were rapidly taken up into phospholipids rather than triacylglycerols. The means by which fatty acids, once taken up by the cell, are transported to the plasma membrane is examined in the next section of this review.

FATE OF ASSIMILATED UNSATURATED FATTY ACID

Researchers have exploited the anaerobically-induced auxotrophic requirement of *Sacccharomyces cerevisiae* for an unsaturated fatty acid and a sterol to supplement the yeast with a range of different unsaturated fatty acids and sterols (Thomas *et al.*, 1978; Thomas and Rose, 1979). These workers have shown $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ fatty-acyl residues to occur in the plasma membrane of *Saccharomyces cerevisiae*. However, once taken up by the yeast, the mechanisms by which the unsaturated fatty acids are packaged and transported to the plasma membrane are unclear. It is likely, however that plasma membrane assembly and secretion share a common structural basis in eucaryotes (Palade ,1975). Secretory proteins are synthesized in the rough ER, are passed onto the Golgi, where they are packaged into vesicles with a membraneous exterior and directed to the cell surface, where they fuse with the membrane externalising their contents. The temperature-sensitive secretory mutants of the yeast *Saccharomyces cerevisiae* X2180, isolated by Novick and Schekman (1979), have provided researchers with a tool for examining whether such a pathway exist in this microbe. The mutants contain conditionally lethal mutations which block the secretory process at a restrictive temperature (37°C) causing accumulation of membraneous organelles and therefore accumulation of proteins being exported for secretion. Many secretory mutants have been isolated and they block at a variety of points within the secretory pathway. Such mutants may block the secretory process before translocation across the ER membrane (Deshaises

and Schekman, 1987), before glycolysation in the ER lumen (Feldman *et al.*, 1987), in the ER membrane (Novick *et al.*, 1981), in the Golgi apparatus (Novick *et al.*, 1980) and at the stage of production of secretory vesicles (Novick and Schekman, 1979). Export of secretory and plasma membrane proteins is completely blocked in all *sec* mutants (Novick and Schekman, 1983). The movement of soluble and membrane bound proteins is believed to be along a secretory pathway in which the sequence of events is: ER → Golgi → vesicles → cell surface or vacuolar compartments, leading to extracellular secretion or vacuolar assembly (Novick *et al.*, 1981).

Most recently Schwenke (1990) has proposed a general pathway which specifically transfers cell-surface components. The postulate involves the following sequence of events. Initially, biosynthesis of the protein and oligosaccharide components of the macromolecule occurs, followed by enclosure of these in membrane-bound structures. Secondly, membrane components are synthesized and assembled, vesicularisation takes place, and vesicles are differentiated and directed to specific membrane sites. Thirdly, macromolecules destined for external or internal secretion are processed, and finally fusion of vesicles occurs with the specific membrane.

Much research over the last decade has examined whether separate secretory pathways exist for the secretion of extracellular molecules and proteins (e. g. mannan, invertase, acid phosphatase) and plasma membrane components. New polarised plasma membrane synthesis in budding cells is achieved, at least in part, by fusion of secretory vesicles (Field and Schekman, 1980). Novick and Schekman (1983) demonstrated an intimate coupling between the secretion of soluble materials and the assembly of plasma membrane proteins, since both required the same SEC gene product for fusion to occur with the plasma membrane. It was postulated that either, plasma membrane proteins and secretory proteins were transported in the same vesicles, or the two types of protein are sorted away from each other and transported in separate vesicles. It would appear that the former hypothesis is correct because

Holcomb *et al.* (1988) demonstrated that plasma membrane Mg^{2+} ATP-ase and the secretory protein, acid phosphatase, are transported by the same vesicle species. Yeast plasma membrane expansion is prevented in *sec* mutants at restrictive temperatures, but phospholipid synthesis is not, suggesting that lack of membrane growth is due to a blockage in the secretory process rather than in a deficiency in a specific membrane component (Ramirez *et al.*, 1983).

Various reports of vesicles in yeasts have appeared in the literature. These have been shown to contain a variety of enzyme activities including exo- and endoglucanase (Matile *et al.*, 1971; Cortat *et al.*, 1972), vacuolar alkaline phosphatase (Schwenke, 1977), acid phosphatase, chitin synthase, mannan synthetase (Cortat *et al.*, 1973) and β-galactosidase (Cartledge and Lloyd, 1972). Some of these vesicles have been shown to contain lipids (Clausen *et al.*, 1974; Cartledge *et al.*, 1977; Schaffner and Matile, 1981). Triacylglycerols and sterol esters appear to be the principal lipid components, accounting for 90-95% of the total lipid content. Vesicles have been shown to be associated with the yeast plasma membrane (Henschke *et al.*, 1983). Henschke and co-workers (1983) isolated membranes and associated vesicles from a randomly dividing population of *Saccharomyces cerevisiae*. It was suggested that vesicles are involved in the transfer of lipids to the plasma membrane prior to their conversion into membrane lipids. It has been reported that transfer of phospholipids was not blocked in the same ways, and not located in the same vesicles, as other envelope materials and secretory proteins in secretory deficient *sec* mutants (Daum *et al.*, 1986). It would appear, therefore, that specific vesicle species maybe involved in the transfer of lipids to areas of membrane expansion, and that others are involved in the transfer of other envelope components.

AIMS OF THE PROJECT

This part of the study examined the means by which exogenously supplied unsaturated fatty acid (in addition to sterol) was taken up by yeast during anaerobic growth.

METHODS

ORGANISMS

The yeasts used in this study were *Saccharomyces cerevisiae* Y185, a haploid yeast strain supplied by J.R. Woodward, and *Saccharomyces cerevisiae* X2180-1Aa, supplied by R.Schekman. The cultures were maintained at 4°C on slopes containing (l^{-1}): malt extract (lab m) 3.0 g; yeast extract (lab m) 3.0 g; glucose 10.0 g; peptone (lab m) 5.0 g and agar 20.0 g (MYGP; Wickerham, 1951).

EXPERIMENTAL CULTURES

Organisms were grown anaerobically in batch culture by a modification of the method of Alterthum and Rose (1973). *Saccharomyces cerevisiae* X2180-1Aa was grown in medium containing (l^{-1}): glucose 50.0 g; yeast extract (lab m) 1.0 or 10.0 g and mycological peptone (lab m) 20.0 g, adjusted to pH 4.5 with concentrated HCl. *Saccharomyces cerevisiae* Y185 was grown in medium containing (l^{-1}): glucose 50.0 g; $(NH_4)_2SO_4$ 3.0 g; KH_2PO_4 4.5 g; yeast extract (lab m) 1.0 g; $MgSO_4.7H_20$ 25 mg and $CaCl_2.2H_2O$ 25 mg, adjusted to pH 4.5 with concentrated HCl. One-litre portions of media were dispensed into 2 l round flat-bottomed flasks and sterilised by autoclaving at 10lb in^{-2} ($6.89x10^4$ Pa) for 5 min. Flasks containing media were fitted to an anerobic system.

To ensure anaerobic conditions nitrogen gas, scrubbed of oxygen by passing through a column-type Oxy-trap (Alltech, Illinois, U.S.A.), was flushed through the flasks for 6 h prior to inoculation. The medium was supplemented with 5 mg ergosterol and 30 mg oleic acid prior to inoculation. Where indicated cultures were supplemented with 2.5 uCi [1-^{14}C] oleic acid either at the start of growth or at various times before harvesting. Medium was inoculated with 1 mg dry weight organisms from an overnight starter culture. Starter cultures (100 ml of the medium used for

growth as already described) were inoculated with a pinhead of organisms from a slant culture and incubated at 30°C for 24 h on an orbital shaker (200 r.p.m.). Experimental cultures were incubated at 30°C with stirring on a flat-bed magnetic stirrer. Growth was followed by measuring the optical density at 600 nm in a LKB Ultrospec 4050 spectrophotometer. Measurements were related to dry weight for each yeast strain using an appropriate calibration curve. Organisms were harvested from mid-exponential phase cultures by centrifugation using a Sorval RC-5 refrigerated Superspeed centrifuge (16000 *g*; 1 min; 4°C; DuPont Co., Delware, U.S.A.) and washed twice with water. Unless otherwise stated, all centrifuge regimes were as already described. Control cultures lacking both ergosterol and oleic acid were incubated with each batch of experimental cultures. If growth in a control culture in defined medium exceeded 0.05 mg dry weight ml^{-1} experimental cultures were rejected.

PREPARATION OF SPHAEROPLASTS

Sphaeroplasts were prepared by a modification of the method of Cartledge *et al.* (1977). Organisms, harvested from cultures of anaerobically-grown *Saccharomyces cerevisiae* Y185 or X2180-1Aa, were washed twice in buffered sorbitol (1.5 M; pH 7.2) containing, 20 mM Tris and 10 mM $MgCl_2$, and resuspended in the same buffer to 10 mg dry weight ml^{-1}. The suspension was supplemented with Zymolyase 100T (Kirin Brewery, Japan) at a rate of 0.1 mg (10 mg dry weight organisms)$^{-1}$ and incubated on an orbital shaker (200 r.p.m.) for 1 h at 30°C. Sphaeroplast formation was followed by diluting 0.1 ml portions of suspension into 2.9 ml of either buffered sorbitol (1.5 M; pH 7.2) or water and measuring the optical density at 600 nm. A Neubauer counting chamber was used to count cells before and after sphaeroplast formation.

ESTIMATION OF OLEIC ACID INCORPORATION BY *SACCHAROMYCES CEREVISIAE* Y185 OR X2180-1Aa

Saccharomyces cerevisiae Y185 or X2180-1Aa was grown anaerobically in the presence of [1-^{14}C] oleic acid. Prior to harvesting, five 3 ml samples of culture were removed and organisms harvested by centrifugation (2000 *g*; 10 min) using an MSE Centaur 2 bench top centrifuge. Organisms were washed twice with water, resuspended in 0.5 ml chloroform-methanol (2:1, v/v) mixture, and transferred to scintillation vials containing 7.5 ml OptiPhase "Safe" scintillant. The remaining organisms were harvested and converted into sphaeroplasts as already desribed. Prior to additon of Zymolyase five 0.5 ml samples were transferred to scintillation vials. After sphareroplasts were obtained they were harvested by centrifugation (3000 *g*; 1 min) and washed three times in buffered sorbitol to remove cell-wall debris. Washed sphaeroplasts were resuspended in 1.5 M sorbitol (25 ml) and five 0.5 ml samples were transferred to scintillation vials. Radioactivity was assayed using a LKB Rackbeta liquid scintillation spectrometer.

A second method of measuring incorporation of [1-^{14}C] oleic acid was to measure the amount of radiolabel associated with washed portions of cells. Portions (1.5 ml) of culture were removed and the cells pelleted by centrifugation (MSE MicroCentaur, 13400 *g*; 2 min). Cells were then washed three times with distilled water before being resuspended in 0.5 ml of water and then assayed for radioactivity as already described.

EXTRACTION OF LIPID

Cells

Organisms from cultures of anaerobically-grown *Saccharomyces cerevisiae* Y185 or X2180-1Aa were harvested, as already described, washed once with water and twice with buffered sorbitol (1.5 M; pH7.2), containing 20 mM Tris and

10 mM $MgCl_2$. Washed cells were treated with hot ethanol (80%, v/v) at 80°C, the suspension mixed, and maintained at that temperature for 15 min in an electrothermal water bath. Lipids were further extracted as already described in the Methods Section of Part One.

Sphaeroplasts

Anaerobically-grown cultures of *Saccharomyces cerevisiae* Y185 or X2180-1Aa were harvested, cells washed and sphaeroplasts formed as already described. Sphaeroplasts were harvested, washed (1100 *g*; 2 min; 4°C) with buffered sorbitol (1.5 M; pH 7.2), and treated with hot ethanol as for cells. Lipids were further extracted from sphaeroplasts as already described in the Methods Section of Part One.

Lipid extracts from cells and sphaeroplasts of *Saccharomyces cerevisiae* Y185 or X2180-1Aa, grown anaerobically in the presence of [1-^{14}C] oleic acid, were separated into individual lipid classes by thin-layer chromatography as described in the methods used in trub fermantations. Lipid bands were marked with a pencil and scraped off into separate scintillation vials containing 7.5 ml OptiPhase "Safe" scintillation fluid. Radioactivity was assayed using an LKB 1217 Rackbeta liquid scintillation spectrometer. The proportion of radioactivity per band was determined.

MATERIALS

All chemicals used were AnalaR grade or of the highest purity available. Lipid standards and supplements were obtained from Sigma Chemical Co. Ltd., Poole, England. Radiolabelled oleic acid was obtained from Amersham International, Amersham, England. Nitrogen gas was supplied by BOC Ltd., Guildford, England.

RESULTS

GROWTH OF *SACCHAROMYCES CEREVISIAE* UNDER ANAEROBIC CONDITIONS

Saccharomyces cerevisiae Y185 grown in defined medium under anaerobic conditions, in the presence of ergosterol and oleic acid, entered the exponential phase of growth after 8 h and approached the stationary phase after 22-24 h (Figure 36). The generation time was 2 h 8 min and cultures reached the mid-exponential phase of growth after approximately 14 h. Varying the amount of oleic acid (20 or 30 mg l^{-1}) added to the medium did not affect the final growth yield which approximated to 0.7 mg dry wt ml^{-1}. The initial decrease in dry wt apparent in Figure 36 may have been due to the slow dispersal of supplements causing a slight cloudiness which gave a measurable response on the spectrophotometer. However, cells from cultures supplemented with 30 mg oleic acid absorbed a greater amount of the acid (Figure 37). The time-course of uptake of oleic acid into *Saccharomyces cerevisiae* Y185 closely mirrored that of growth.

A second strain of *Saccharomyces cerevisiae*, namely X2180-1Aa, grown under anaerobic conditions, in YEPD medium containing ergosterol and oleic acid, entered the exponential phase of growth after 10 h (Figure 38). The generation time was 2 h 30 min and cultures reached the mid-exponential phase of growth after 17 h 30 min. The growth yield was approximately 8.0 mg dry wt ml^{-1} after 30 h at which point the stationary phase of growth had not been reached. In the presence of ergosterol, *Saccharomyces cerevisiae* X2180-1Aa entered the exponential phase of growth after 8 h and approached the stationary phase of growth after 25 h. The generation time was 2 h 15 min and the culture reached the mid-exponential phase of growth after 16 h. The final growth yield was approximately 4.0 mg dry wt ml^{-1}. In the presence of oleic acid, strain X2180-1Aa entered the exponential phase of growth after 10 h and approached the stationary phase of growth after 20 h. The mid-

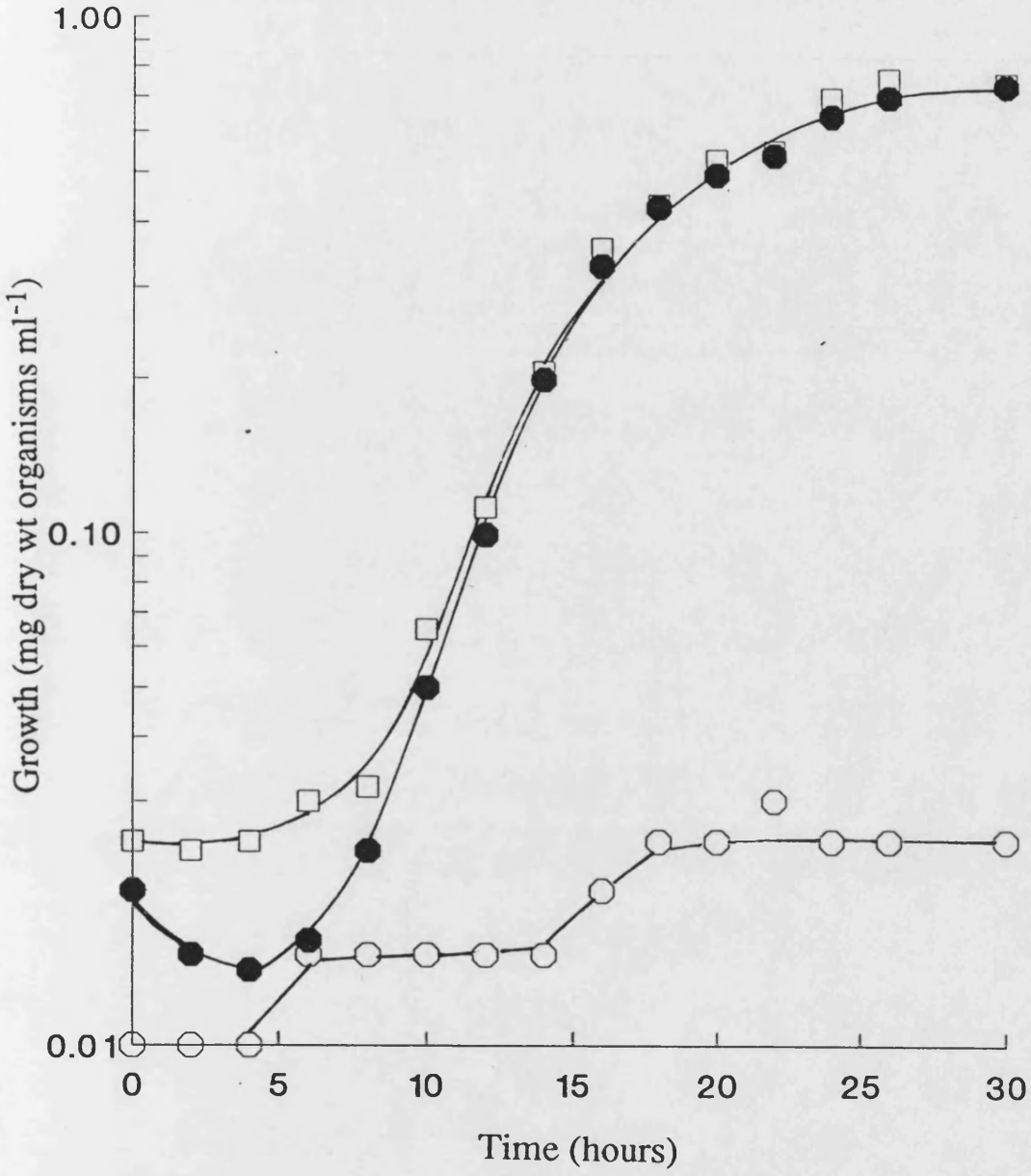


FIGURE 36. Time-course of growth of *Saccharomyces cerevisiae* Y185 in defined medium under anaerobic conditions. Values plotted are the average of three independent determinations and the variation never exceeded the 5% confidence limit. Key: (◯) no supplement control; (●) 5 mg ergosterol l^{-1} and 20 mg [1-^{14}C] oleic acid l^{-1} supplements; (□) 5 mg ergosterol l^{-1} and 30 mg [1-^{14}C] oleic acid l^{-1} supplements.

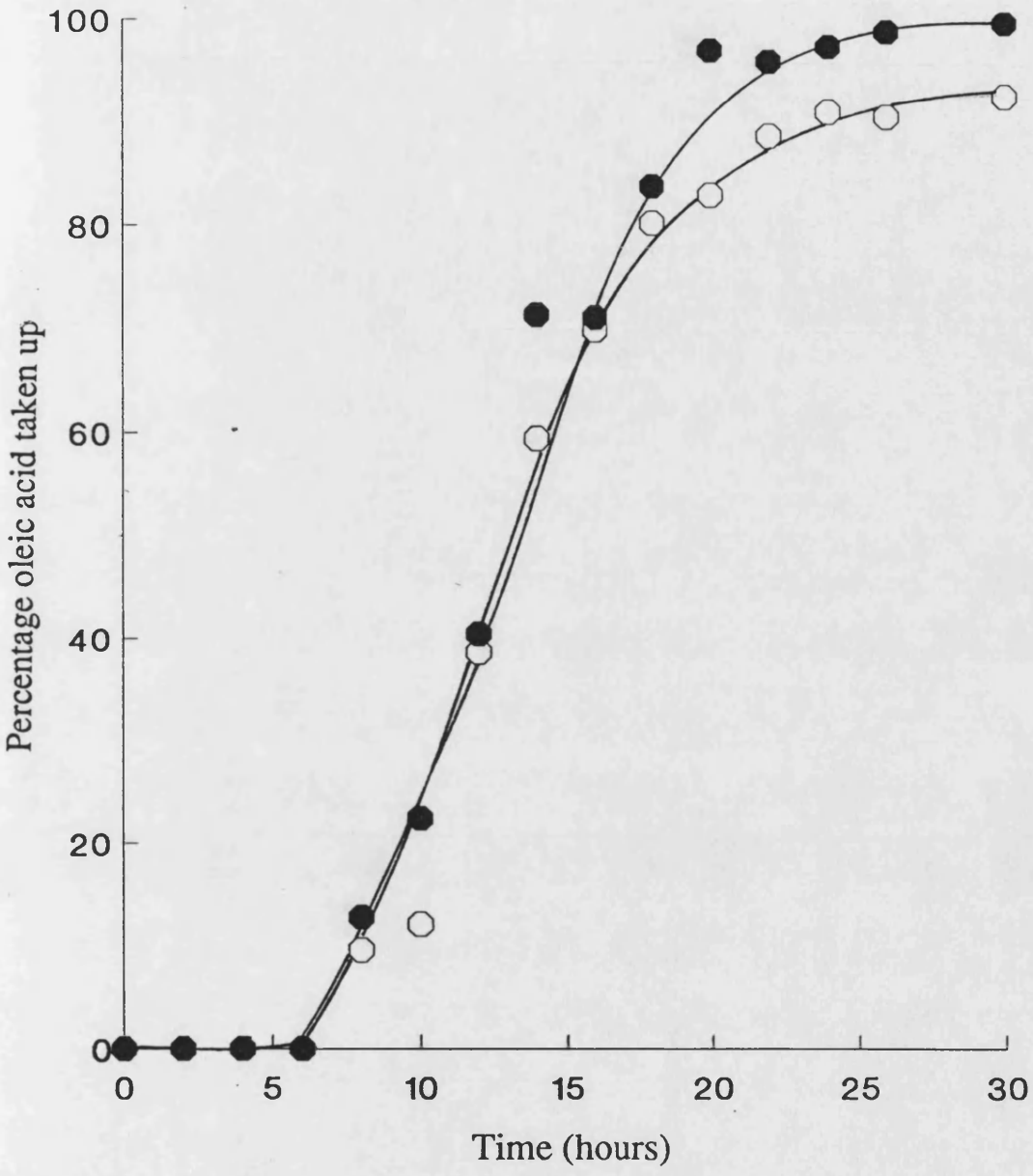


FIGURE 37. Time-course of uptake of [1-^{14}C] oleic acid by *Saccharomyces cerevisiae* Y185 grown in defined medium under anaerobic conditions. Values plotted are the average of three independent determinations and the variation never exceeded the 5% confidence limit. Key: (◯) 5 mg ergosterol l^{-1} and 20 mg [1-^{14}C] oleic acid l^{-1} supplements; (●) 5 mg ergosterol l^{-1} and 30 mg [1-^{14}C] oleic acid l^{-1} supplements.

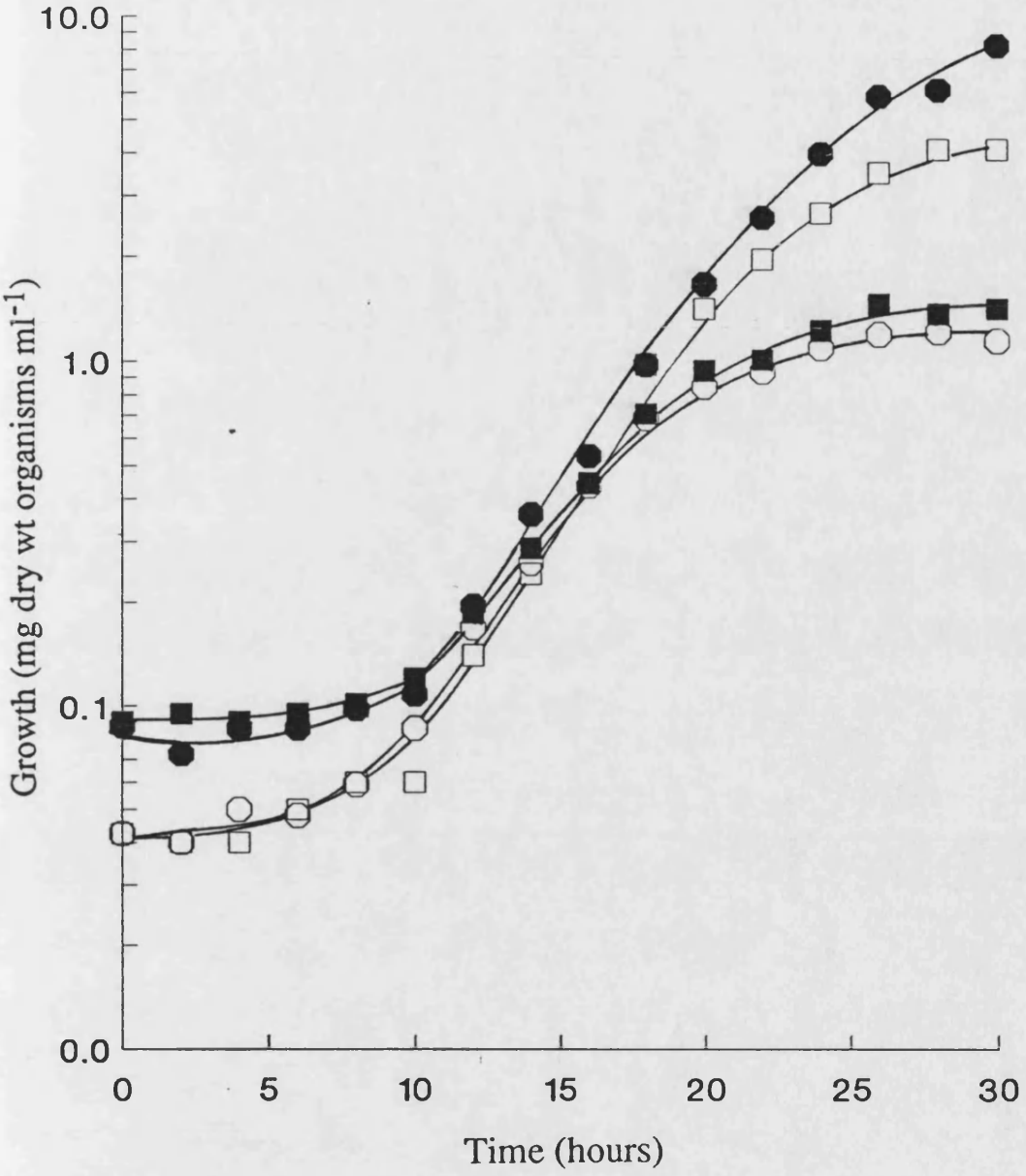


FIGURE 38. Time-course of growth of *Saccharomyces cerevisiae* X2180-1Aa in YEPD medium under anaerobic conditions. Values plotted are the average of three independent determinations and the variation never exceeded the 5% confidence limit. Key: (◯) no supplement control; (■) 5 mg ergosterol l^{-1} supplement; (□) 30 mg oleic acid l^{-1} supplement; (●) 5 mg ergosterol l^{-1} and 30 mg [1-^{14}C] oleic acid l^{-1} supplements.

exponential phase of growth was reached after 15 h 30 min and the generation time was 2 h 45 min. Cultures that were not supplemented entered the exponential phase of growth after 8 h and approached the stationary phase of growth after 25 h. The mid-exponential phase of growth was reached after 14 h and the generation time was 2 h 45 min. *Saccharomyces cerevisiae* X2180-1Aa grown anaerobically in the presence of 10 mg or 20 mg oleic acid and ergosterol entered the exponential phase of growth after 8 h (Figure 39). Cultures supplemented with 10 mg oleic acid approached the stationary phase of growth after 25 h, whereas those supplemented with 20 mg had only approached this growth phase after 30 h (Figure 39). The generation time was 2 h 30 min in both types of culture and the mid-exponential phase of growth was reached after 16 h 45 min. The final yield was approximately 5.5 mg dry wt ml^{-1} for cultures supplemented with 10 mg oleic acid and approximately 6.5 mg dry wt ml^{-1} for cultures supplemented with 20 mg of the acid. Cultures that were not supplemented entered the exponential phase of growth after 8 h and approached the stationary phase of growth after 22 h. The generation time was 2 h 30 min and the mid-exponential phase of growth was reached after 15 h 15 min. The final yield was approximately 1.5 mg dry wt ml^{-1}. Uptake of oleic acid, measured as the percentage present in the cells of that supplied, was greatest in cells from cultures of strain X2180-1Aa supplemented with 30 mg oleic acid (Figure 40). Cells from stationary-phase cultures supplemented with 10 mg or 20 mg oleic acid had only taken up 68 and 74%, respectively, of the available oleic acid.

INCORPORATION OF OLEIC ACID INTO ANAREOBICALLY-GROWN *SACCHAROMYCES CEREVISIAE*

At the mid-exponential phase of growth cells of anaerobically-grown *Saccharomyces cerevisiae* Y185 had taken up 62.1 ± 2.4% of the supplied oleic acid, whereas cells of *Saccharomyces cerevisiae* X2180-1Aa had taken up 73.1 ± 2.4%. The amount of oleic acid associated with organisms decreased on conversion to

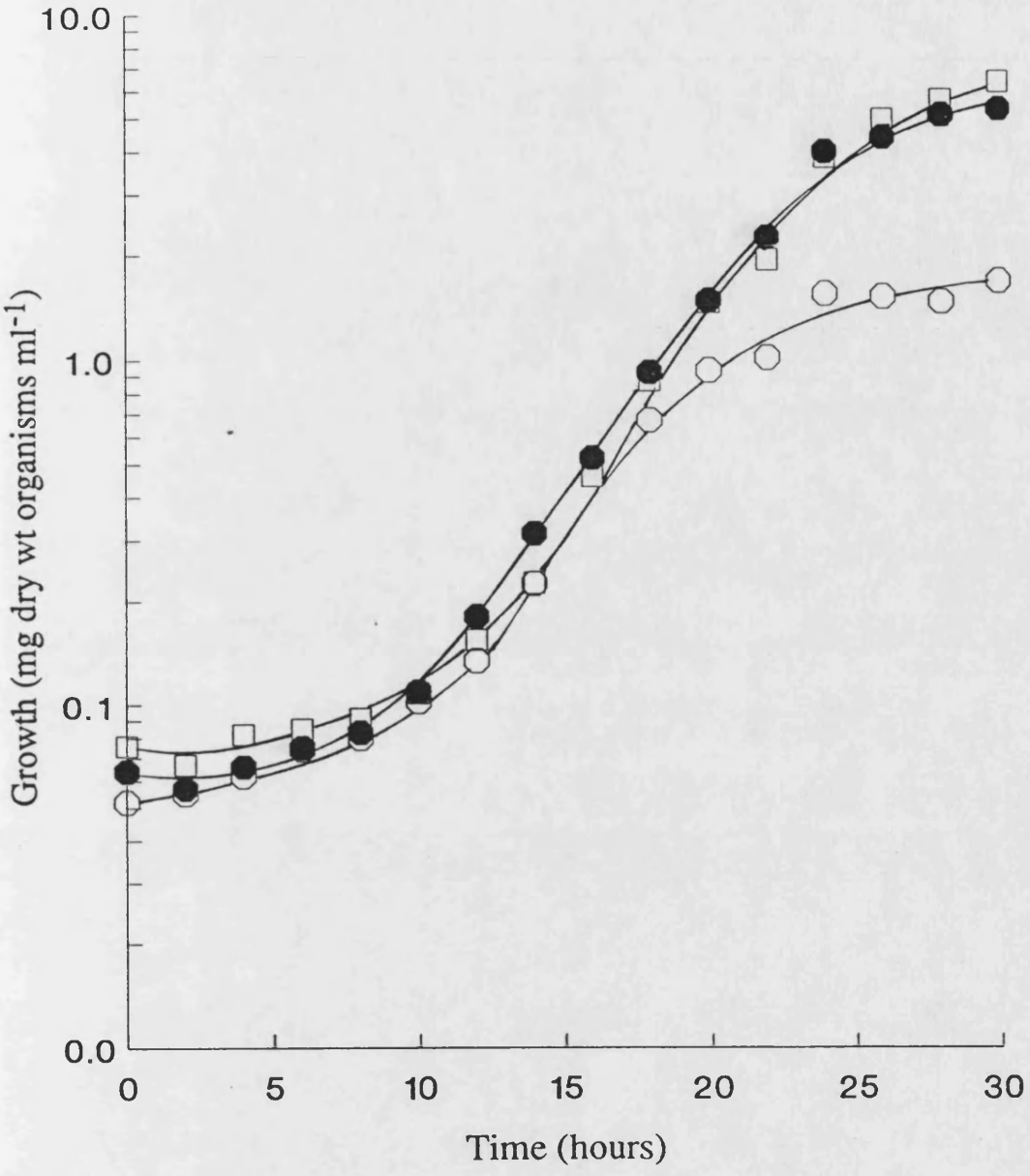


FIGURE 39. Time-course of growth of *Saccharomyces cerevisiae* X2180-1Aa in YEPD medium under anaerobic conditions. Values plotted are the average of three independent determinations and the variation never exceeded the 5% confidence limit. Key: (◯) no supplement control; (●) 5 mg ergosterol l^{-1} and 10 mg [1-^{14}C] oleic acid l^{-1} supplements; (□) 5 mg ergosterol l^{-1} and 20 mg [1-^{14}C] oleic acid l^{-1} supplements.

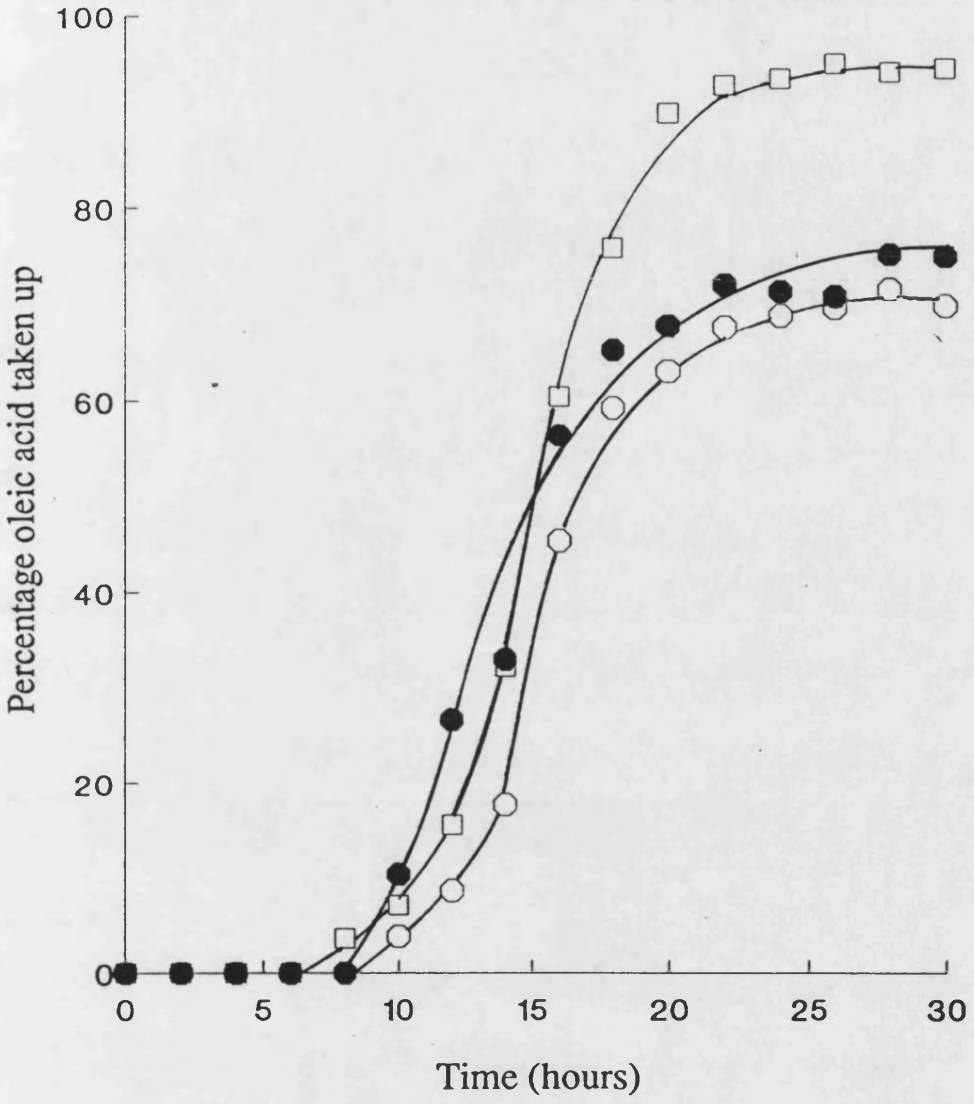


FIGURE 40. Time-course of uptake of [1-^{14}C] oleic acid by *Saccharomyces cerevisiae* X2180-1Aa grown in YEPD medium under anaerobic conditions. Values plotted are the average of three independent determinations and the variation never exceeded the 5% confidence limit. Key: (○) 5 mg ergosterol l^{-1} and 10 mg [1-^{14}C] oleic acid l^{-1} supplements; (●) 5 mg ergosterol l^{-1} and 20 mg [1-^{14}C] oleic acid l^{-1} supplements; (□) 5 mg ergosterol l^{-1} and 30 mg [1-^{14}C] oleic acid l^{-1} supplements.

sphareoplasts, to 55.7 ± 3.2% for *Saccharomyces cerevisiae* Y185, and to 67.3 ± 2.8% for *Saccharomyces cerevisiae* X2180-1Aa, respectively.

INCORPORATION OF OLEIC ACID INTO LIPIDS OF ANAEROBICALLY-GROWN *SACCHAROMYCES CEREVISIAE*

Lipid extracts from cells of *Saccharomyces cerevisiae* Y185 grown anaerobically in the presence of oleic acid and harvested from the mid-exponential phase of growth contained a high proportion of free oleic acid (Table 35). About half of the oleic acid taken up by the yeast was incorporated into phospholipids while only 5.6 and 1.0% was incorporated into triacylglycerols and sterol esters. When cells were converted into sphaeroplasts, the distribution of radiolabel in lipid classes changed considerably (Table 35), with a decrease in the proportion of oleic-acid residues in the free fatty acid and phospholipids classes. However, the amount of label in the triacylglycerol and sterol-ester classes increased. Arcsin transformations and t tests were used to detect significant differences ($p = 0.05$) in the size of lipid classes between cells and sphaeroplasts. The proportions of radiolabel found in the free fatty acid, sterol-ester and triacyglycerol classes in cells were significantly different from those found in sphaeroplasts.

Inhibitor studies

Various inhibitors were used to try to prevent the change in radiolabelled oleic acid distribution between cells and sphaeroplasts. Addition of a mixture of chloramphenicol (10 mg l^{-1}), *para*-chloromercuribenzoic acid (50 mM) and cycloheximide (10 mg l^{-1}) to cultures 15 min prior to harvesting had a profound effect on the proportion of free oleic-acid residues in both cells and sphaeroplasts (Table 36). A greater porportion of free oleic-acid residues were present in cells and sphaeroplasts from cultures to which the inhibitor mixture had been added than in those to which it was not (Tables 35 and 36). There was a redistribution of oleic-acid

TABLE 35. Distribution of oleic-acid residues amongst lipid classes in extracts from cells, and sphaeroplasts derived from these cells, from anaerobically-grown *Saccharomyces cerevisiae* Y185. Cultures were harvested at the mid-exponential phase of growth. Values quoted are the average of four independent determinations ± standard deviation.

Lipid class	Percentage oleic acid present in	
	Cells	Sphaeroplasts
Phospholipids	50.8 ± 5.9	48.1 ± 4.9
Diacylglycerols	3.1 ± 0.8	4.3 ± 1.8
Free fatty acids	39.4 ± 3.7	11.8 ± 4.3
Triacylglycerols	5.6 ± 1.8	27.8 ± 6.9
Sterol esters	1.0 ± 0.3	8.0 ± 3.2

TABLE 36. Distribution of oleic-acid residues amongst lipid classes in extracts from cells, and sphaeroplasts derived from these cells, of anaerobically-grown *Saccharomyces cerevisiae* Y185. Cultures were treated with a mixture of chloramphenicol (10 mg l^{-1}), *para*-chloromercuribenzoic acid (50 mM) and cycloheximide (10 mg l^{-1}) dissolved in 10 ml 2 M NaOH. The inhibitor cocktail was added to cultures 15 min prior to harvesting at the mid-exponential phase of growth . Values quoted are the average of four independent determinations ± standard deviation.

Lipid class	Percentage oleic acid present in	
	Cells	Sphaeroplasts
Phospholipids	12.9 ± 7.1	6.5 ± 2.4
Diacylglycerols	0.5 ± 0.03	1.3 ± 0.08
Free fatty acids	81.9 ± 7.8	71.7 ± 10.2
Triacylglycerols	2.9 ± 0.4	18.9 ± 1.3
Sterol esters	1.9 ± 0.8	1.2 ± 0.1

residues from the free form into triacylglycerols when sphaeroplasts were formed. However, the percentage difference was not so great as that for cultures not containing the inhibitors. The proportion of radiolabel in phospholipids in both cells and sphaeroplasts was lowered to 12.9 and 6.5%, respectively, compared with 50.8 and 48.1% in cultures not containing the inhibitors. Addition of 10 μM nocodazole (methyl [5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl]-carbamate) did not prevent accumulation of oleic-acid residues in triacylglycerols and sterol esters when sphaeroplasts were formed (Table 37). The proportion of oleic-acid residues detected in sterol esters and triacylglycerols in sphaeroplasts was significantly greater ($p = 0.05$) than that in cells. However, the proportion of oleic-acid residues present in the free fatty acids in sphaeroplasts was significantly lower ($p = 0.05$) than that in cells. A similar pattern occurred when 100 μM colchicine was used (Table 38). When sphaeroplasts were formed, there was a significant decrease ($p = 0.05$) in the proportion of oleic-acid residues present in diacylglycerols and free fatty acids compared with cells. There was a significant increase ($p = 0.05$) in the proportion of oleic-acid residues present in both the sterol-ester and triacylglycerol classes in sphaeroplasts. When 1 mM cycloheximide was used, similar results were obtained (Table39). A significant decrease ($p = 0.05$) in the proportion of free fatty acids and a significant increase in the proportion of sterol esters and triacylglycerols occurred when sphaeroplasts were formed. Lipid extracts from cells of *Saccharomyces cerevisiae* X2180-1Aa, grown anaerobically in the presence of oleic acid and harvested from the mid-exponential phase of culture growth, contained a large amount of oleic-acid residues in phospholipids (Table 40). Unlike the Y185 strain, the X2180-1Aa strain did not contain a great amount of free fatty acid. After formation of sphaeroplasts there was a significant ($p = 0.05$) decrease in the proportion of phospholipid and a significant increase in the proportion of triacylglycerol present.

TABLE 37. Distribution of oleic-acid residues amongst lipid classes in extracts from cells, and sphaeroplasts derived from these cells, from anaerobically-grown *Saccharomyces cerevisiae* Y185. Cultures were treated with a mixture of chloramphenicol (10 mg l^{-1}), cycloheximide (10 mg l^{-1}) and nocodazole (10μM) dissolved in 10 ml 2 M NaOH. The inhibitor cocktail was added to cultures 15 min prior to harvesting at the mid-exponential phase of growth. Values quoted are the average of four independent determinations ± standard deviation.

Lipid class	Percentage oleic acid present in	
	Cells	Sphaeroplasts
Phospholipids	54.4 ± 4.1	51.5 ± 3.1
Diacylglycerols	1.9 ± 0.4	2.5 ± 0.6
Free fatty acids	34.0 ± 6.1	7.5 ± 1.1
Triacylglycerols	8.3 ± 2.2	28.8 ± 3.2
Sterol esters	1.2 ± 0.3	9.7 ± 1.1

TABLE 38. Distribution of oleic-acid residues amongst lipid classes in extracts from cells, and sphaeroplasts derived from these cells, from anaerobically-grown *Saccharomyces cerevisiae* Y185. Cultures were treated with a mixture of chloramphenicol (10 mg l^{-1}), colchicine (100 μM) and cycloheximide (10 mg l^{-1}) dissolved in 10 ml 2 M NaOH. The inhibitor cocktail was added to cultures 15 min prior to harvesting at the mid-exponential phase of growth. Values quoted are the average of four independent determinations ± standard deviation.

Lipid class	Percentage oleic acid present in	
	Cells	Sphaeroplasts
Phospholipids	56.8 ± 12.9	42.5 ± 6.3
Diacylglycerols	3.4 ± 0.4	1.0 ± 0.1
Free fatty acids	29.0 ± 10.7	4.5 ± 3.3
Triacylglycerols	10.5 ± 2.7	50.5 ± 8.1
Sterol esters	0.4 ± 0.1	1.4 ± 0.5

TABLE 39. Distribution of oleic-acid residues amongst lipid classes in extracts from cells, and sphaeroplasts derived from these cells, from anaerobically-grown *Saccharomyces cerevisiae* Y185. Cultures were treated with a mixture of chloramphenicol (10 mg l^{-1}) and cycloheximide (1 mM) dissolved 10 ml in 2 M NaOH. The inhibitor cocktail was added to cultures 15 min prior to harvesting at the mid-exponential phase of growth. Values quoted are the average of three independent determinations ± standard deviation.

Lipid class	Percentage oleic acid present in	
	Cells	Sphaeroplasts
Phospholipids	67.6 ± 13.6	68.1 ± 4.6
Diacylglycerols	6.0 ± 0.9	6.0 ± 2.4
Free fatty acids	21.6 ± 4.9	2.0 ± 1.2
Triacylglycerols	4.1 ± 1.1	22.4 ± 3.2
Sterol esters	0.6 ± 0.4	2.1 ± 0.3

TABLE 40. Distribution of oleic-acid residues amongst lipid classes in extracts from cells, and sphaeroplasts derived from these cells, from anaerobically-grown *Saccharomyces cerevisiae* X2180-1Aa. Cultures were harvested at the mid-exponential phase of growth. Values quoted are the average of four independent determinations ± standard deviation.

Lipid class	Percentage oleic acid present in	
	Cells	Sphaeroplasts
Phospholipids	72.2 ± 2.6	49.7 ± 5.1
Diacylglycerols	5.0 ± 0.7	1.7 ± 0.7
Free fatty acids	3.4 ± 0.9	1.4 ± 0.7
Triacylglycerols	18.7 ± 3.2	45.9 ± 8.8
Sterol esters	0.4 ± 0.1	1.4 ± 0.4

Pulse-label studies

Cells of *Saccharomyces cerevisiae* Y185 were grown under anaerobic conditions with ergosterol and oleic acid supplements as described in the Methods section. Oleic acid was added at various times prior to harvesting the cells at mid-exponential phase. Lipid extracts were prepared from washed cells and radioactivity assayed as already described in the Methods section. Initially oleic acid was taken up in the free form and as the time between pulsing and harvesting increased oleic acid was incorporated into other lipid classes (Table 41). However, after pulsing with oleic acid for 1 h, the greatest proportion of fatty acid was in the free form (40.9%), with incorporation into phospholipids and triacylglycerols accounting for most of the rest.

TABLE 41. Distribution of radiolabel into lipid classes in extracts from cells of anaerobically-grown *Saccharomyces cerevisiae* Y185. Cultures were pulse-labelled with [1-^{14}C] oleic acid at 5, 10, 20, 40 and 60 min prior to harvesting. Cultures were harvested at the mid-exponential phase of growth. Values quoted are the average of three independent determinations ± standard deviation.

Lipid class	Duration of pulse-labelling and percentage of oleic acid in each class				
	5 min	10 min	20 min	40 min	60 min
Phospholipids	5.0 ± 0.5	6.6 ± 1.3	9.7 ± 4.3	17.1 ± 3.9	32.0 ± 6.1
Monoacyl-glycerols	1.2 ± 0.9	0.7 ± 0.1	6.4 ± 1.8	8.2 ± 3.9	0.7 ± 0.2
Diacylglycerols	0.3 ± 0.1	0.3 ± 0.1	2.8 ± 1.3	3.4 ± 1.1	2.5 ± 1.0
Free fatty acids	91.4 ± 1.4	87.2 ± 1.9	78.7 ± 9.6	66.7 ± 7.7	40.9 ± 5.6
Triacylglycerols	1.5 ± 0.2	2.9 ± 1.7	4.4 ± 3.0	2.7 ± 0.8	23.2 ± 1.4
Sterol esters	0.4 ± 0.1	2.5 ± 1.5	0.7 ± 0.3	1.0 ± 0.4	0.6 ± 0.1

DISCUSSION

For a number of years the Zymology Laboratory at the University of Bath has been interested in compositon-function relationships in the plasma membrane of *Saccharomyces cerevisiae*. By exploiting the anaerobically-induced requirement of *Saccharomyces cerevisiae* for a sterol and an unsaturated fatty acid, plasma membranes have been enriched in specific supplied sterols and fatty-acyl residues. However, little is known about how exogenous fatty acids are incorporated into the plasma membrane of yeast.

INCORPORATION OF OLEIC ACID INTO *SACCHAROMYCES CEREVISIAE*

In this study, the growth characteristics of *Saccharomyces cerevisiae* Y185, grown under strict anaerobic conditions in media supplemented with oleic acid and ergosterol were in agreement with previous reports using this yeast (Calderbank *et al.*, 1984, 1985; White, 1987). Using anaerobically-grown *Saccharomyces cerevisiae* Y185, White (1987) found that organisms harvested from the mid-exponential phase of growth (an arbitrary point that has been used for the study of lipid biochemistry and physiology in the yeast plasma membrane) had incorporated approximately 50% of the supplied fatty acid. In this study *Saccharomyces cerevisiae* Y185 had incorporated over 60% of the supplied fatty acid, whereas *Saccharomyces cerevisiae* X2180-1Aa had incorporated over 70%. These differences could be attributed to the different growth characteristics of the two yeast strains. Removal of the cell wall from both strains of yeast decreased the amount of oleic acid associated with organisms. This may be due to the removal of free oleic acid bound to the cell wall (Kohlwein and Paltauf, 1983). Alternatively, this loss may be due to removal of oleic-acid residues bound to the glycerolipids of the cell wall (Nurminen and Suomolainen, 1969; Suomolainen and Nurminen, 1970). Since yeast cell wall preparations are often

contaminated with plasma membrane fragments the former is the more likely explanation.

Varying the amount of oleic acid supplemented to anaerobically-grown *Saccharomyces cerevisiae* Y185 did not alter the final growth yield or the time taken for it to be achieved. This implies that the oleic acid supplement, at the concentrations being used in these experiments, was not the primary factor in limiting yeast growth. However, decreasing the amount of oleic acid supplemented to anaerobically-grown cultures of *Saccharomyces cerevisiae* X2180-1Aa decreased the amount of yeast mass produced, although the generation time was unaffected. It is apparent that 30 mg of oleic acid supplement allows greater growth by prolonging the period of time that strain X2180-1Aa is in the exponential-phase of growth. It is interesting to note that anaerobically-grown cultures of *Saccharomyces cerevisiae* X2180-1Aa not supplemented with ergosterol and oleic acid had a growth yield of 1.5 mg (dry wt ml)$^{-1}$. This suggests that a component(s) of the YEPD medium acts as a source of a sterol and / or an unsaturated fatty acid. Another interesting point concerned the growth of strain X2180-1Aa under anaerobic conditions when supplemented with either ergosterol or oleic acid. Supplementation with the former led to a greater amount of yeast growth implying that, either YEPD medium acts as a source of oleic acid or that *Saccharomyces cerevisiae* X2180-1Aa has a greater requirement for a sterol rather than an unsaturated fatty acid. A further point to consider was the finding that cells from anaerobically-grown cultures of *Saccharomyces cerevisiae* X2180-1Aa supplemented with 30 mg of oleic acid took up a greater proportion of the supplement than cells from cultures supplemented with 10 or 20 mg of the acid. Why this should be so is not entirely clear, although it may account for the poorer yeast mass yields from the latter fermentations. Similarly, cells from cultures of anaerobically-grown *Saccharomyces cerevisiae* Y185 supplemented with 30 mg of oleic acid took up a greater proportion of the supplement than those supplemented with 20 mg. However, the amounts of yeast mass produced were the same.

It is apparent, at least in the case of anaerobically-grown cultures of

Saccharomyces cerevisiae X2180-1Aa, that altering the amount of oleic acid supplemented, while keeping the ergosterol supplement constant, prolongs the duration of the exponential-phase of growth. The next part of this discussion deals briefly with the fate of exogenously supplied oleic acid.

INCORPORATION OF OLEIC ACID INTO LIPDS OF ANAEROBICALLY-GROWN *SACCHAROMYCES CEREVISIAE*

The results of this study confirm those of White (1987) who demonstrated a large difference in the distribution of oleic-acid residues in lipids from cells and sphareoplasts of *Saccharomyces cerevisiae* Y185. These results substantiate the theory that lipid metabolism occurs during sphaeroplast formation. The most notable feature of lipids from *Saccharomyces cerevisiae* Y185 was the prevalence of free oleic acid. The occurrence of large amounts of free oleic acid in yeast cells is somewhat surprising. It is generally assumed that free fatty acids are toxic to yeast cells and their appearance in lipid extracts is considered an artefact (Ratledge and Evans, 1987). However, a variation of the hot ethanol extraction method of Letters (1968) was used to extract lipids. The hot ethanol denatures lipases and phospholipases and, as such, the detection of free oleic acid in lipid extracts from cells of *Saccharomyces cerevisiae* Y185 is not due to the enzymatic degradation of phospholipids and triacylglycerols. It can only be concluded that *Saccharomyces cerevisiae* Y185 is able to tolerate high intracellular concentrations of free fatty acid. However, in cells of *Saccharomyces cerevisiae* X2180-1Aa the supplemented oleic acid was found predominantly in phospholipids and, to a lesser degree, triacylglycerols. On conversion of cells to sphaeroplasts there was transfer of oleic-acid residues from phospholipids to triacylglycerols in strain X2180-1Aa, whereas in strain Y185 free oleic acid was transferred to triacylglycerols. Many researchers have suggested that triacylglycerols may serve to regulate fatty-acyl residues found in plasma membrane phospholipids (Haley and Jack, 1977; Watson and Rose, 1980).

Taylor and Parks (1978, 1979) have shown that triacylglycerols and sterol esters accumulate in the late-exponential phase and early stationary phase of growth in cultures of *Saccharomyces cerevisiae*. White (1987) proposed that the transfer of oleic acid from its free form into triacylglycerols in *Saccharomyces cerevisiae* Y185 was due to an induced stationary phase of growth during sphaeroplast preparation. The same theory would account for the transfer of oleic-acid residues from phospholipids to triacylglycerols during the conversion of cells of *Saccharomyces cerevisiae* X2180-1Aa to sphaeroplasts.

It is evident that, when cells are converted to sphaeroplasts, lipid metabolism occurs in strains of *Saccharomyces cerevisiae*. It is also clear that none of the inhibitors used in this study were effective, at the concentrations used, in preventing intracellular transfer of free oleic acid into triacylglycerols in *Saccharomyces cerevisiae* Y185. The inhibitors used were chloramphenicol, *para*-chloromercuribenzoic acid, colchicine, cycloheximide and nocodazole. Chloramphenicol and cycloheximide inhibit protein synthesis and might be expected to arrest cell growth, whereas *para*-chloromercuribenzoic acid inhibits lipase activity. Colchicine and nocodazole inhibit the assembly of microtubules, which have been implicated in intracellular transport mechanisms in cells of higher organisms (Birkett *et al.*, 1981; Havercroft *et al.*, 1981; Williams and Lee, 1976). It is likely that microtubules are involved in the secretory and transport pathways, and hence plasma membrane growth, in yeast cells. In the case of colchicine this is not altogether surprising since Haber *et al.* (1972) found that it did not arrest growth of *Saccharomyces cerevisiae*. It is presumed that the transfer of free oleic acid to triacylglycerols on conversion of cells to sphaeroplasts would be via an ordered pathway, requiring vesicularisation and transport mediated by microtubules. However, no evidence exists from the results of these studies to suggest the involvement of microtubules in such a pathway.

The results of the pulse-labelling experiments carried out in this study, using oleic acid and *Saccharomyces cerevisiae* Y185, contrast with those of Kohlwein and

Paltauf (1983). These workers showed that, within 30 s of supplementation, up to 35% of the supplied fatty acids had been taken up by the yeast cell and incorporated into the phospholipids of the *Saccharomyces uvarum (cerevisiae)*. However, such rapid incorporation of fatty acids into phospholipids of *Saccharomyces cerevisiae* Y185 does not occur. As reported earlier in this discussion, *Saccharomyces cerevisiae* Y185 appears to be able to tolerate high concentrations of free fatty acid. This is borne out by the pulse-labelling experiments, in which exogenously supplied oleic acid was absorbed, and presumably stored in the free form, until it was esterified into phospholipids and to a lesser extent monoacylglycerols and triacylglycerols. As the duration of pulsing increased the proportion oleic acid in the free form in cells decreased. However, the evidence from studies conducted with *Saccharomyces cerevisiae* X2180-1Aa, where free oleic acid was not found in significant amounts in cells, and those of Kohlwein and Paltauf (1983), suggest that *Saccharomyces cerevisiae* Y185 is atypical in its ability to store exogenously supplied fatty acid in the free form.

CONCLUSIONS

The findings of this study suggest that the fate of unsaturated fatty acids supplemented to anaerobically-grown cultures of *Saccharomyces cerevisiae* is strain dependent. It is apparent that *Saccharomyces cerevisiae* Y185 is atypical in its lipid metabolism due to its ability to accumulate large quantities of free oleic acid. As such *Saccharomyces cerevisiae* Y185 does not lend itself to studies investigating the fate of exogenously supplied unsaturated fatty acids in anaerobically-grown yeast. The change in distribution of oleic acid in lipids on conversion of cells to sphaeroplasts occurred in both strains of *Saccharomyces cerevisiae* studied. The transfer was from free oleic acid to triacylglycerols in *Saccharomyces cerevisiae* Y185, and from phospholipids to triacylglycerols in *Saccharomyces cerevisiae* X2180-1Aa. This finding has important repercussions where the study of the mechanisms governing the

uptake and incorporation of exogenously supplied unsaturated fatty acids in yeast cells is concerned. The use of secretory mutants of *Saccharomyces cerevisiae* (Schekman, 1982) may provide a tool for investigating the fate of unsaturated fatty acids supplied to anaerobically-grown *Saccharomyces cerevisiae*. However, elucidation of the sub-cellular location of fatty acids requires, amongst other things, that cells be converted to sphareoplasts and the evidence provided in this thesis shows that lipid metabolism continues through such conversion. A more thorough study of the inhibitors available may discover an inhibitor that can arrest such intracelluar lipid transfer. It is clear that this intracellular transfer of lipids within *Saccharomyces cerevisiae* must be arrested if information on the fate of exogenously supplied fatty acids is have any meaning.

REFERENCES

AHVENAINEN, J. (1982) *Journal of the Institute of Brewing* **88**, 367.

AHVENAINEN, J. (1983) Technical Research Centre of Finland, Publication **12**, 1.

AKBAR, M.D., RICKARD, P.A.D. & MOSS, F.J. (1974) *Biotechnology and Bioengineering* **16**, 455.

AKIN, C. & KRABBE, E. (1966) Proceedings of the American Society of Brewing Chemists p.86.

ALTERTHUM, F. & ROSE, A.H. (1973) *Journal of General Microbiology* **77**, 371.

ANDERSON, R.G. & KIRSOP, B.H. (1974) *Journal of the Institute of Brewing* **80**, 48.

ANDERSON, R.G. & KIRSOP, B.H. (1975) *Journal of the Institute of Brewing* **81**, 111.

ANDREASEN, A.A. & STIER, T.J.B. (1953) *Journal of Cellular and Comparative Physiology* **41**, 23.

ANDREASEN, A.A. & STIER, T.J.B. (1954) *Journal of Cellular and Comparative Physiology* **43**, 271.

ANNESS, B.J. (1984) *Journal of the Institute of Brewing* **90**, 315.

ANNESS, B.J. & REED, R.J.R. (1985 a) *Journal of the Institute of Brewing* **91**, 82.

ANNESS, B.J. & REED, R.J.R. (1985 b) *Journal of the Institute of Brewing* **91**, 313.

ARIES, V. & KIRSOP, B.H. (1977) *Journal of the Institute of Brewing* **83**, 220.

ARIES, V. & KIRSOP, B.H. (1978) *Journal of the Institute of Brewing* **84**, 118.

ARKINA, V. (1968) *Monatsschrift für Brauerei* **21**, 247.

ÄYRÄPÄÄ, T. (1971) *Journal of the Institute of Brewing* **77**, 266.

ÄYRÄPÄÄ, T. & LINDSTROM, I. (1977) Proceedings of the European Brewery Convention Congress (Amsterdam) p.507.

BAMFORTH, C.W. (1981) Proceedings of the European Brewery Convention Congress (Copenhagen) p.335.

BAMFORTH, C.W. & JACKSON, G. (1983) Proceedings of the European Brewing Convention Congress (London) p.331.

BEAVAN, M.J., CHARPENTIER, C. & ROSE, A.H. (1982) *Journal of General Microbiology* **128**, 1447.

BIRKETT, C.R., COULSON, C., POGSON, C.I. & GULL, K. (1981) *Biochemical Pharmacology* **30**, 1629.

BLIGH, E.G. & DYER, W.J. (1959) *Canadian Journal of Biochemistry* **37**, 911.

BLOCH, K.E. (1983) *CRC Critical Reviews of Biochemistry* **14**, 47.

BLOOMFIELD, D.K. & BLOCH, K. (1958) *Biochimica et Biophysica Acta* **30**, 220.

BLOOMFIELD, D.K. & BLOCH, K. (1960) *Journal of Biological Chemistry* **235**, 337.

BRADFORD, M.M. (1976) *Analytical Biochemistry* **72**, 248.

BROWN, C.M. & HOUGH, J.S. (1965) Proceedings of the European Brewing Convention Congress (Stockholm) p. 223.

BROWN, M.L. & KIRSOP, B.H. (1972) *Journal of the Institute of Brewing* **78**, 39.

BROWN, S.W., OLIVER, S.G., HARRISON, D.E.F & RIGHELATO, R.C. (1981) *European Journal of Applied Microbiology and Biotechnology* **11**, 151.

BULDER, C.J.E.A. & REININK, M. (1974) *Antonie van Leeuwenhoek* **40**, 445.

CALDERBANK, J., KEENAN, M.H.J., ROSE, A.H. & HOLMAN, G.D. (1984) *Journal of General Microbiology* **130**, 2817.

CALDERBANK, J., KEENAN, M.H.J., ROSE, A.H. (1985) *Journal of General Microbiology* **131**, 57.

CANTRELL, I.C. & ANDERSON, R.G. (1983) Proceedings of the European Brewing Convention Congress, (London) p.481.

CARRINGTON, R., COLLETT, R.C., RUNKIN, I.R. & HALEK, G. (1972) *Journal of the Institute of Brewing* **78**, 243.

CARTLEDGE, T.G., ROSE, A.H., BELK, D.M. & GOODHALL, A.A. (1977) *Journal of Bacteriology* **132**, 426.

CARTLEDGE, T.G. & LLOYD, D. (1972) *Biochemical Journal* **126**, 755.

CARTWRIGHT, C.P., JUROSEK, J.R., BEAVAN, M.J., RUBY, F.M.S., DE MORAIS, S.M.F. & ROSE, A.H. (1986) *Journal of General Microbiology* **132**, 369.

CASEY, G.P. & INGLEDEW, W.M. (1983) *Journal of the American Society of Brewing Chemists* **41**, 148.

CASEY, G.P. & INGLEDEW, W.M. (1985) *Journal of the American Society of Brewing Chemists* **43**, 75.

CASEY, G.P., MAGNUS, C.A. & INGLEDEW, W.M. (1983) *Biotechnology Letters* **5**, 429.

CASEY, G.P., MAGNUS, C.A. & INGLEDEW, W.M. (1984) *Applied and Environmental Microbiology* **48**, 639.

CASTELLI, A., LITTARRU, G.P. & BARBARESI, G. (1969) *Archiv für Mikrobiologie* **66**, 34.

CHEN, C.H. (1980) Proceedings of the American Society of Brewing Chemists **38**, 148

CHOW, S.L. & HOLLANDER, D. (1978) *Lipids* **13**, 768.

CLAPPERTON, J.F. & BROWN, D.G.W. (1978) *Journal of the Institute of Brewing* **84**, 90.

CLAPPERTON, J.F., DALGLEISH, C.E. & MEILGAARD, M.C. (1976) *Journal of the Institute of Brewing* **82**, 7.

CLAUSEN, M.K., CHRISTIANSEN, K. JENSON, P.K. & BEANKE, O. (1974) *FEBS Letters* **43**, 176.

COLEMAN, P.L. & WEINER, H. (1973) *Biochemistry* **12**, 3466.

CORTAT, M., MATILE, P. & WIEMKEN, A. (1972) *Archiv für Mikrobiologie* **82**, 189.

CORTAT, M., MATILE, P. & KOPP, F. (1973) *Biochemical and Biophysical Research Communications* **53**, 482.

CURTAIN, C.C., ATWELL, J.L., LOONEY, F.D. & ZAJAC-CADE, A. (1985) Proceedings of the Convention of the Institute of Brewing (Australia and New Zealand Section) p. 236.

D'AMORE, T., PANCHAL, C.J. & STEWART, G.G. (1987) *Journal of the Institute of Brewing* **93**, 472.

D'AMORE, T., PANCHAL, C.J. & STEWART, G.G. (1988) *Applied and Environmental Microbiology* **54**, 110.

D'AMORE, T., PANCHAL, C.J., RUSSELL, I. & STEWART, G.G. (1990) *CRC Critical Reviews in Biotechnology* **9**, 287.

DASARI, G., RODDICK, F., CONNOR, M.A. & PAMMENT, N.B. (1983) *Biotechnology Letters* **5**, 715.

DASARI, G., RODDICK, F., CONNOR, M.A. & PAMMENT, N.B. (1990) *Biotechnology and Bioengineering* **35**, 109.

DAUM, G., HEIDORN, E. & PALTAUF, F. (1986) *Biochimica et Biophysica Acta* **878**, 93.

DAVELOOSE, M. (1987) *Technical Quarterly of the Master Brewers Association of America* **24**, 109.

DAVID, M.H. (1974) *Journal of the Institute of Brewing* **80**, 80.

DAVID, M.H. & KIRSOP, B.H. (1972) Proceedings of the American Society of Brewing Chemists p.14.

DAVID, M.H. & KIRSOP, B.H. (1973 b) *Journal of the Institute of Brewing* **79**, 20.

DAY, A., ANDERSON, E. & MARTIN, P.A. (1975) Proceedings of the European Brewing Convention Congress, (Nice) p.377.

DESHAISES, R.J. & SCHEKMAN, R. (1987) *Journal of Cell Biology* **105**, 633.

DELENTE, J. & GURLEY, J. (1968) *Technical Quarterly of the Master Brewers Association of America* **5**, 187.

DELENTE, J., AKIN, C., KRABBE, K. & LADENBURG,K. (1969) *Biotechnology and Bioengineering* **9**, 631.

DIAMOND, R.J. & ROSE, A.H. (1970) *Journal of Bacteriology* **102**, 311.

DOMBEK, K.M. & INGRAM, L.O. (1984) *Journal of Bacteriology* **157**, 233.

DOMBEK, K.M. & INGRAM, L.O., (1986 a) *Applied and Enviromental Microbiology* **51**, 197.

DOMBEK, K.M. & INGRAM, L.O. (1986 b) *Applied and Environmental Microbiology* **52**, 975.

DOMBEK, K.M. & INGRAM, L.O. (1988) *Applied and Environmental Microbiology* **54**, 98.

DROST, B.W., VAN EERDE, P., HOEKSTRA, S.F. & STRATING, J. (1971) Proceedings of the European Brewery Convention Congress, (Estoril) p.451.

FELDMAN, R.I., BERNSTEIN, M. & SCHEKMAN, R. (1987) *Journal of Biological Chemistry* **262**, 9332.

FIELD, C. & SCHEKMAN, R. (1980) *Journal of Cell Biology* **86**, 123.

FOLCH, J., LEES, M. & SLOANE-STANLEY, G.H. (1957) *Journal of Biological Chemistry* **226**, 497.

FRERMAN, F.E. & BENNET, W. (1973) *Archives of Biochemistry and Biophysics* **159**, 434.

FRYBERG, M., OEHLSCHLAGER, A.C. & UNRAU, A.M. (1973) *Journal of the American Chemical Society* **95**, 5747.

FUHRMANN, G.F. & ROTHSTEIN, A. (1968) *Biochimica et Biophysica Acta* **163**, 325.

GOTTLIEB, S.F. (1971) *Annual Review of Microbiology* **25**, 111.

GRAY, W.D. (1948) *Journal of Bacteriology* **55**, 53.

GRUNWALD-RAJI, H. & MARGALITH, P. (1990) *Journal of Applied Bacteriology* **68**, 247.

GUIJARRO, J.M. & LAGUNAS, R. (1984) *Journal of Bacteriology* **160**, 874.

HABER, J.E., PELOQUIN, J.G., HALVORSON, H.O. & BORISY, G.G. (1972) *Journal of Cell Biology* **55**, 355.

HACKSTAFF, B.W. (1978) *Technical Quarterly of the Master Brewers Association of America* **15**, 1.

HAHN-HAEGARDAHL, B. (1982) *Applied Biochemistry and Biotechnology* **7**, 43.

HALEY, J.E. & JACK, R.C. (1977) *Biochimica et Biophysica Acta* **489**, 207.

HARRIS, G., BARTON-WRIGHT, E.C, & CURTIS, N.S. (1951) *Journal of the Institute of Brewing* **57**, 264.

HAUKELI, A.D. & LIE, S. (1976) *Journal of the Institute of Brewing* **82**, 161.

HAVERCROFT, J.C., QUINLAN, R.A. & GULL, K. (1981) *Journal of Cell Science* **49**, 195.

HAWTHORNE, D.B. JONES, R.D. BARETT, P.A., KAVANAGH, T.E. & CLARKE, B.J. (1986) *Journal of the Institute of Brewing* **92**, 181.

HAYASHIDA, S. & OHTA, K. (1978) *Agricultural and Biological Chemistry* **42**, 1139.

HAYASHIDA, S. & OHTA, K. (1980) *Agricultural and Biological Chemistry* **44**, 2561.

HAYASHIDA, S., FENG, D.D. & HONGO, M. (1974) *Agricultural and Biological Chemistry* **38**, 2001.

HAYASHIDA, S., FENG, D.D. & HONGO, M. (1975) *Agricultural and Biological Chemistry* **39**, 1025.

HAYASHIDA, S., FENG, D.D., OHTA, K., CHAITIUMVONG, S. & HONGO, M. (1976) *Agricultural and Biological Chemistry* **40**, 73.

HELIN, T.R.M. & SLAUGHTER, J.C. (1977) *Journal of the Institute of Brewing* **83**, 17.

HENSCHKE, P.A., THOMAS, D.S., ROSE, A.H. & VEAZEY, F.J. (1983) *Journal of General Microbiology* **129**, 2927.

HOLCOMB, C.L., HANSEN, W.J., ETCHEVERRY, T. & SCHEKMAN, R. (1988) *Journal of Cell Biology* **106**, 641.

HOLZMANN, A. & PIENDL, A. (1976) *Journal of the American Society of Brewing Chemists* **35**, 1.

HOLZBERG, I., FINN, R.K. & STEINKRAUS, K.H. (1967) *Biotechnology and Bioengineering* **9**, 413.

HONGO, M., HAYASHIDA, S., INOUE, S., KOIZUM, R. & KAWAHARADA, H. (1967) *Journal of the Agricultural and Chemical Society* **41**, 629.

HOSSACK, J.A. & ROSE, A.H. (1976) *Journal of Bacteriology* **127**, 67.

HOUGH, J.S. BRIGGS, D.E., STEVENS, R. & YOUNG, T.W. (1982) " Malting and Brewing Science", Vol. 2, p. 524, Chapman and Hall, London and New York.

HUDSON, J.R. (1959) *Journal of the Institute of Brewing* **65**, 321.

HUDSON, J.R. (1973) Proceedings of the European Brewery Convention Congress, (Salzburg), p.157.

HUNTER, K. & ROSE, A.H. (1971) In "The Yeasts", (Rose, A.H. & Harrison, J.S. Eds.), Vol 2, p. Academic Press, London and New York.

HUNTER, K. & ROSE, A.H. (1972) *Biochimica et Biophysica Acta* **260**, 639.

INGLEDEW, W.M. & KUNKEE, R.E. (1985) *American Journal of Enology and Viticulture* **36**, 65.

INOUE, T. (1975) *Reports of the Research Laboratories, Kirin Brewery Co Ltd.* **18**, 13.

INOUE, T., TAKAOKA, Y. & HA'A, S. (1962) *Journal of Fermentation Technology* **40**, 511.

ISHIZAKI, A., SHIBAI, H., HIROSE, Y. & SHIRO, T. (1971) *Agricultural and Biological Chemistry* **35**, 1733.

JACOBSEN, T., LIE, S. & HAGE, T. (1981) Proceedings of the European Brewing Convention Congress (Copenhagen) p.97.

JAHNKE, L. & KLEIN, H.P. (1983) *Journal of Bacteriology* **155**, 488.

JOLLOW, D., KELLERMAN, G.M. & LINNANE, A.W. (1968) *Journal of Cell Biology* **37**, 221.

JONES, M. & PIERCE, J.S. (1964) *Journal of the Institute of Brewing* **70**, 307.

JONES, M.D., COPE, R. & RAINBOW, C. (1975) Proceedings of the European Brewing Convention Congress (Nice) p.669.

JONES, R.P. & GREENFIELD (1981) *Biotechnology Letters* **3**, 225.

JONES, R.P. & GREENFIELD, P.F. (1982) *Enzyme and Microbial Technology* **4**, 210.

JONES, R.P. & GREENFIELD, P.F. (1984 a) *Process Biochemistry* **19**, 48.

JONES, R.P. & GREENFIELD, P.F. (1984 b) *Biotechnology Letters* **6**, 471.

JONES, R.P. & GREENFIELD, P.F. (1987 a) *Yeast* **3**, 223.

JONES, R.P. & GREENFIELD, P.F. (1987 b) *Enzyme and Microbial Technology* **9**, 334.

JONES, R.P., PAMMENT, N & GREENFIELD P.F. (1981) *Process Biochemistry* **16**, 42.

KALMOKOFF, M.L. & INGLEDEW, W.M. (1985) *Journal of the American Society of Brewing Chemists* **43**, 189.

KANELLAKI, M., KOUTINAS, A.A., KANA, K., NICOLOPOULOU, M., PAPADIMITRIOU, A. & LYCOURGHCOTIS, A. (1989) *Biotechnology and Bioengineering* **34**, 121.

KATES, M. & HAGEN, P.O. (1964) *Canadian Journal of Biochemistry* **42**, 481.

KEENAN, M.H.J. & ROSE, A.H. (1979) *FEMS Microbiology Letters* **6**, 133.

KEENAN, M.H.J., ROSE, A.H. & SILVERMAN, B.W. (1982) *Journal of General Microbiology* **128**, 2547.

KIRSOP, B.H. (1974) *Journal of the Institute of Brewing* **80**, 252.

KIRSOP, B.H. (1977) *Technical Quarterly of the Master Brewers Association of America* **14**, 227.

KIRSOP, B.H. (1978) *Brewers Digest* (July) p.28.

KIRSOP, B.H. (1982) In "Topics in Enzyme and Fermentation Biotechnology" (Wiseman, A.,Ed) Vol.6 p.79.

KLEIN, H.P. (1955) *Journal of Bacteriology* **69**, 620.

KLEIN, H., EATON, N. & MURPHY, J. (1954) *Biochimica et Biophysica Acta* **13**, 591.

KLEIN, K., STEINBERG, R., FIETHEN, B., & OVERATH, P. (1970) *European Journal of Biochemistry* **19**, 442.

KLOPPER, W. K., TUNING, B. & VERMIERE, H.A. (1975) Proceedings of the European Brewing Convention Congress (Nice) p.659.

KNATCHBULL, F.B. & SLAUGHTER, J.C. (1987) *Journal of the Institute of Brewing* **93**, 420.

KOHLWEIN, S.P. & PALTAUF, F. (1983) *Biochimica et Biophysica Acta* **792**, 310.

KRUGER, E. (1971) *Monatsschrift für Brauerei* **24**, 268.

KUNKEE, R.E. & OUGH, C.S. (1966) *Applied Microbiology* **14**, 643.

LAFON-LAFOURCADE, S., GENEIX, C. & RIBÉREAU-GAYON, P. (1979) *Applied and Environmental Microbiology* **38**, 1069.

LAFON-LAFOURCADE, S., GENEIX, C. & RIBÉREAU-GAYON, P. (1984) *Applied and Environmental Microbiology* **47**, 1246.

LARUE, F., LAFON-LAFOURCADE, S. & RIBÉREAU-GAYON (1984) *Biotechnology Letters* **6**, 687.

LEAO, C. & VAN UDEN, N. (1982) *Biotechnology and Bioengineering* **24**, 2601.

LEAO, C. & VAN UDEN, N. (1983) *Biotechnology and Bioengineering* **25**, 2085.

LEAO, C. & VAN UDEN, N. (1984a) *Biotechnology and Bioengineering* **26**, 403.

LEAO, C. & VAN UDEN, N. (1984b) *Biochimica et Biophysica Acta* **774**, 43.

LENTINI, A., JONES,R.D., WHEATCROFT,R., LIM,Y.H., FOX, C., HAWTHORNE, D.B. & KAVANAGH, T.E. (1990) Proceedings of the Convention of the Institute of Brewing (Australia and New Zealand Section) p.158.

LETTERS, R. (1968) In "Aspects of Yeast Metabolism" (Mills, A.K., Ed.) Blackwell Press, Oxford.

LIE, S., HAUKELI, A.D. & JACOBSEN, T. (1975) Proceedings of the European Brewing Convention Congress (Nice) p.601.

LIGHT, R.J., LENNARZ, W.J. & BLOCH, K. (1962) *Journal of Biological Chemistry* **237**, 1793.

LOUGHREY, B.M. & LETTERS, R. (1983) Proceedings of the European Brewing Convention Congress (London) p.659.

LOUREIRO, V. & FERREIRA, H.G. (1983) *Biotechnology and Bioengineering* **25**, 2263.

LOUREIRO-DIAS, M.C. & PEINADO, J.M. (1982) *Biotechnology Letters* **4**, 721.

LUMSDEN, W.B., DUFFUS, J.H. & SLAUGHTER, J.C. (1987) *Journal of General Microbiology* **133**, 877.

MACLEOD, A.M. (1977) In "Economic Microbiology" (Rose, A.H., Ed.), Vol.1. p.43, Academic Press London and New York.

MACLEOD, A.M. & MILLAR, A.S. (1962) *Journal of the Institute of Brewing* **68**, 322.

MACLEOD, A.M. & PALMER, G.H. (1966) *Journal of the Institute of Brewing*, **72**, 580.

MACPHERSON, J.K. & BUCKEE, G.K. (1974) *Journal of the Institute of Brewing* **80**, 540.

MACY, J.M. & MILLER, M.W. (1983) *Archives of Microbiology* **134**, 64.

MADDOX, I.S. & HOUGH, J.S (1970) *Journal of the Institute of Brewing* **76**, 262.

MASSCHELEIN, C.A. (1981) Monograph VII. European Brewery Convention Flavour Symposium, p.211.

MATILE, P., CORTAT, M. & WIEMKEN, A. (1971) Proceedings of the National Academy of Science USA **68**, 638.

McMURROUGH, I. & ROSE, A.H. (1967) *Biochemical Journal* **105**, 189.

MEILGAARD, M.C., DALGLEISH, C.E. & CLAPPERTON, J.F. (1979) *Journal of the Institute of Brewing* **85**, 38.

MERRITT, N.R. (1967) *Journal of the Institute of Brewing* **73**, 484.

MILLAR, D.G., GRIFFITHS-SMITH, K., ALGAR, E. & SCOPES, R.K (1982) *Biotechnology Letters* **4**, 601.

MINISTRY OF AGRICULTURE FISHERIES AND FOOD (MAFF) (1986) "The Analysis of Agricultural Materials", 3rd Edn., pp. 8, 36, HMSO, London.

MITZ, M.A. (1979) *Journal of Theoretical Biology* **80**, 537.

MORRISON, W.R., TAN, S.L. & HARGIN, R.D. (1980) *Journal of the Science of Food and Agriculture* **31**, 329.

MOTA, M., STREHAIANO, P., & GOMA, G. (1984) *Journal of the Institute of Brewing* **90**, 360.

MUNOZ, E. & INGLEDEN, W.M. (1989 a) *American Journal of Enology and Viticulture* **40**, 61.

MUNOZ, E. & INGLEDEN, W.M. (1989 b) *Applied and Environmental Microbiology* **55**, 1560.

NABIAS, R.C., SA-CORREIA, I., VIEGAS, C.A. & NOVAIS, J.M. (1988) *Applied and Environmental Microbiology* **54**, 2439.

NAGODAWITHANA, T. & STEINKRAUS, K.H. (1976) *Applied and Environmental Microbiology* **31**, 158.

NES, W.R. (1974) *Lipids* **9**, 596.

NES, W.R. & MCKEAN, M.L. (1977) In "Biochemistry of Steroids and other Isopentenoids" University Park Press (Baltimore).

NORTON, J.S. & KRAUSS, R.W. (1972) *Plant and Cell Physiology* **13**, 139.

NAVARRO, J.M. & DURRAND, G. (1978) *Annals of Microbiology (Paris)* **129B**, 215.

NOVAK, M., STREHAIANO, P., MORENO, M. & GOMA, G. (1981) *Biotechnology and Bioengineering* **23**, 201.

NOVICK, P. & SCHEKMAN, R. (1979) Proceedings of the National of Academy of Science USA **76**, 1858.

NOVICK, P. & SCHEKMAN, R. (1983) *Journal of Cell Biology* **96**, 541.

NOVICK, P., FIELD, C. & SCHEKMAN, R. (1980) *Cell* **21**, 205.

NOVICK, P., FERRO, S. & SCHEKMAN, R. (1981) *Cell* **25**, 461.

NUNN, W.D., SIMONS, R.W., EGAN, P.A. & MALOY, S.R. (1979) *Journal of Biological Chemistry* **254** 9130.

NURMINEN, T. & SUOMALAINEN, H. (1969) *Antonie van Leeuwenhoek (supplements)* **35**, 83.

NURMINEN, T., KONTTINEN, K. & SUOMOLAINEN, H. (1975) *Chemistry and Physics of Lipids* **14**, 15.

NURMINEN, T., TASKINEN, T. & SUMOLAINEN, H. (1976) *Biochemical Journal* **154**, 751.

O'CONNOR-COX, E.S.C. & INGLEDEW, W.M. (1990) *Journal of the American Society of Brewing Chemists* **48**, 26.

OHNO, T. & TAKAHASHI, R. (1986 a) *Journal of the Institute of Brewing* **92**, 84.

OHNO, T. & TAKAHASHI, R. (1986 b) *Journal of the Institute of Brewing* **92**, 88.

OHTA, K. & HAYASHIDA, S. (1983) *Applied and Environmental Microbiology* **46**, 821.

OLSEN, B.H. & JOHNSON, M.J. (1949) *Journal of Bacteriology* **57**, 235.

O'PALKA, J., EIDET, I. & JACKSON, L.L. (1987) *Journal of Food Science* **52**, 515.

OUGH, C.S. & GROAT, M.L. (1978) *Applied and Environmental Microbiology* **35**, 881.

OURA, E., HAARASILTA, S. & LONDESBOROUGH, J. (1980) *Journal of General Microbiology* **118**, 51.

OWADES, J.L. (1981) *Technical Quarterly of the Master Brewers Association of America* **18**, 163.

PALADE, G. (1975) *Science* **189**, 347.

PALMER, G.H. (1974) *Journal of the Institute of Brewing* **80**, 13.

PANCHAL, C. J. & STEWART, G.G. (1980) *Journal of the Institute of Brewing* **86**, 207.

PANCHAL, C.J., PEACOCK, L. & STEWART, G.G. (1982) *Biotechnology Letters* **4**, 639.

PASTEUR, L. (1866) "Études sur le Vin" Masson, Paris.

PASTEUR, L. (1876) "Études sur la Bière" Gauthier-Villars, Paris.

PFISTERER, E. & STEWART, G.G. (1975) Proceedings of the European Brewing Convention Congress (Nice) p.255.

POLLOCK, J.R.A. (1988) *Fermentation* **1**, 31.

POPJAK, G. & CORNFORTH, J.W. (1960) *Advances in Enzymology* **22**, 281.

PRASAD, R. & ROSE, A.H. (1986) *Yeasts* **2**, 205.

PRICE, M.L., VAN SCOYOC, S. & BUTLER, L.G. (1978) *Journal of Agricultural Food Chemistry* **26**, 1214.

PROUDLOCK, J.W., WHEELDON, L.W., JOLLOW, D.J. & LINNANE, A.W. (1968) *Biochimica et Biophysica Acta* **152**, 434.

RAMIREZ, R.M., ISHIDA-SCHICK, T., KRILOWICZ, B.L., LEISH, B.A. & ATKINSON, K.D. (1983) *Journal of Bacteriology* **154**, 1276.

RATLEDGE, C. & EVANS, C.T. (1987) In "The Yeasts" (Rose, A.H. and Harrison, J.S., Eds.) Vol.4 Academic Press, London and New York.

RIBÉREAU-GAYON, P. (1985) *American Journal of Enology and Viticulture* **36**, 1.

RICE, J.F., HELBERT, J.R. & GARVER, J.C. (1974) Proceedings of the American Society of Brewing Chemists p.94.

RICE, J.F., CHICOYE, E., HELBERT, J.R. & GARVER, J.C. (1977) *Journal of the American Society of Brewing Chemists* **35**, 35.

RODRíGUEZ-NAVARRO, A. & SANCHO, E.D. (1979) *Biochimica et Biophysica Acta* **552**, 322.

ROMAN, G.N., JANSEN, N.B. & TSAO, G.T. (1984) *Biotechnology Letters* **6**, 7.

ROSE, A.H. (1979) Monograph V European Brewing Convention Fermentation and Storage Symposium (Zoeterwoude), p.96.

ROSE, A.H. (1980 a) In "Biology and Activity of Yeasts" (Skinner, F.F., Passmore, S.M. and Davenport, P.R., Eds.) Academic Press, London and New York.

ROSE, A.H. (1980 b) *Society for Applied Bacteriology* **9**, 103.

ROSE, A.H. & HARRISON, J.S. (1990) In "The Yeasts" (Rose, A.H. and Harrison, J.S., Eds.) Vol. 5, Academic Press, London and New York.

SAFE, S. (1974) *Biochimica et Biophysica Acta* **326**, 471.

SAMUEL, D., PARIS, S. & AILHAUD, G. (1976) *European Journal of Biochemistry* **64**, 583.

SCHAFFNER, G. & MATILE, P. (1981) *Biochemie und Physiologie der Pflanzen* **176**, 659.

SCHAUS, O.O. (1971) *Technical Quarterly of the Master Brewers Association of America* **8**, 7.

SCHEKMAN, R. (1982) *Trends in Biochemical Sciences* **7**, 243.

SCHISLER, D.O., RUCCO, J.J. & MABEE, M.S. (1982) *Journal of the American Society of Brewing Chemists* **40**, 57.

SCHWENKE, J. (1977) *Physiologie Végétale* **15**, 491.

SCHWENKE, J. (1990) In "The Yeasts" (Rose, A.H. and Harrison, J.S., Eds.) Vol. 5, Academic Press, London and New York.

SHOCK, R.A.W. (1982) In "Multiphase Science and Technology Vol.1" (Hewitt, G.F., Delhaye, J.M. and Zuber, N. Eds.) Hemisphere, London p.281.

SIEBERT, K.J., BLUM, P.H., WISK, T.J., STENROOS, L.E. & ANKLAM, W.J. (1986) *Technical Quarterly of the Master Brewers Association of America* **23**, 37.

SLAUGHTER, J.C. (1989) In "Biotechnology Applications in Beverage Production" (Cantarelli, C. and Lanzarini, G., Eds.) Elsevier Applied Science, London and New York, p.49.

SLAUGHTER, J.C., FLINT, P.W.N. & KULAR, K.S (1987) *FEMS Microbiology Letters* **40**, 239.

STACK, J.B., JOE, F.L., CUNNNINGHAM, D.G., FAZIO, T. & ROACH, J.A.G. (1986) *Journal of the Association of Official Analytical Chemists* **69**, 551.

STEWART, G.G., D'AMORE, T., PANCHAL, C.J. & RUSSELL, I. (1988) *Technical Quarterly of the Master Brewers Association of America* **25**, 47.

STEINKRAUS, K.H., CULLEN, R.E., PAREKH, L.J. & JELEN, V. (1981) In "Biochemical Engineering" (Ghosh, T., Ed.) Vol. 2.

STONE, I. (1962) *Wallerstein Laboratories Communications* **25**, 88.

SUOMOLAINEN, H. & KERANEN, A.J.A. (1968) *Chemistry and Physics of Lipids* **2**, 296.

SUOMOLAINEN, H. & NURMINEN, T. (1970) *Chemistry and Physics of Lipids* **4**, 247.

SUOMOLAINEN, H. & NURMINEN, T. & OURA, E. (1967) *Archives of Biochemistry and Biophysics* **118**, 219.

TAYLOR, F.R. & PARKS, L.W. (1978) *Journal of Bacteriology* **136**, 531.

TAYLOR, F.R. & PARKS, L.W. (1979) *Biochimica et Biophysica Acta* **575**, 204.

TAYLOR, G.T. & KIRSOP, B.H. (1977) *Journal of the Institute of Brewing* **83**, 97.

TAYLOR, G.T., THURSTON, P.A. & KIRSOP, B.H. (1979) *Journal of the Institute of Brewing* **85**, 219,

THOMAS, D.S. & ROSE, A.H. (1979) *Archives of Microbiology* **122**, 49.

THOMAS, D.S., HOSSACK, J.A. & ROSE, A.H. (1978) *Archives of Microbiology* **117**, 239.

THURSTON, P.A., TAYLOR, R. & AHVENAINEN, J. (1981) *Journal of the Institute of Brewing* **87**, 92.

THURSTON, P.A., QUAIN, D.E. & TUBB, R.S. (1982) *Journal of the Institute of Brewing* **88**, 90.

TROLLE, B. (1950) *Journal of the Institute of Brewing* **56**, 364.

VALLEE, B.L. & HOCH, F.L. (1955) *Journal of the American Chemical Society* **77**, 821.

VANDERWINKEL, E. FURMANSKI, P., REEVES, H.C. & ALI, S.J. (1968) *Biochemical and Biophysical Research Communications* **33**, 902.

VAN ENGEL, E.L. (1969) Proceedings of the American Society of Brewing Chemists p.35.

VERNON, P.S. (1985) *Brewers Guardian* **114**, 25.

VIEGAS, C.A., SA-CORREIA, I. & NOVIAS, J.M. (1985) *Applied and Environmental Microbiology* **50**, 1333.

VISURI, K. & KIRSOP, B.H. (1970) *Journal of the Institute of Brewing* **76**, 362.

WACKERBAUER, K. & BENDER, G. (1983) *Monatsschrift für Brauerei* **36**, 152.

WALKER, G.M. & DUFFUS, J.H. (1980) *Journal of Cell Science* **42**, 329.

WALKER-CAPRIOGLIO, H.M., RODRIGUEZ, R.J. & PARKS, L.W. (1985) *Applied and Environmental Microbiology* **50**, 685.

WATSON, K. & ROSE, A.H. (1980) *Journal of General Microbiology* **117**, 225.

WHITE, F.W. (1978) Proceedings of the Convention of the Institute of Brewing (Australia and New Zealand Section) p.133.

WHITE, M.J. (1987) Ph.D. Thesis, University of Bath, England.

WHITEAR, A.L. & CRABB, D. (1977) *The Brewer (Feb)* p.60.

WHITWORTH, C. (1978) Monograph V. European Brewery Convention Fermentation and Storage Symposium (Zoeterwoude), p.155.

WICKERHAM, L.J. (1951) United States Department of Agriculture Technical Bulletin no 1029, Washington D.C.: Department of Agriculture.

WILLAMS, J.A. & LEE, M. (1976) *Journal of Cell Biology* **71**, 795.

ZURCHER, C. (1971) *Monatsschrift für Brauerei* **24**, 276.